AD_____

FURTHER DEVELOPMENT AND VALIDATION OF THE FROG EMBRYO TERATOGENESIS ASSAY - XENOPUS (FETAX)

Midterm Report

John A. Bantle, Ph.D.

May 12, 1989

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-88-C-8031

Oklahoma State University
Department of Zoology
430 Life Sciences West
Stillwater, Oklahoma 74078

Robert A. Finch, Ph.D.
Contracting Officer's Representative
U.S. Army Biomedical Research & Development Laboratory
Fort Detrick, Frederick, MD 21701-5010

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1a REPORT SECURITY CLASSIFICATION Unclassified	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)	5 MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION Oklahoma State University 6b OFFICE SYMBOL (If applicable)	7a NAME OF MONITORING ORGANIZATION U.S. Army Biomedical Research and Development Laboratory
6c ADDRESS (City, State, and ZIP Code) Stillwater Oklahoma 74078	7b ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5010
8a NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Laboratory 8b OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract No. DAMD17-88-C-8031
8c ADDRESS (City, State and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10 SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO: 61102A; PROJECT NO: 61102BS15; TASK NO: CC; WORK UNIT ACCESSION NO: 072

11 TITLE (Include Security Classification)
Further Development and Validation of the Frog Embryo Teratogenesis Assay- Xenopus (FETAX)

12 PERSONAL AUTHOR(S)
Bantle, John A.

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Midterm Report	FROM 9/26/88 TO 3/26/89	1989 May 12	165

16 SUPPLEMENTARY NOTATION

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Frog Embryo Teratogenesis Assay-Xenopus (FETAX) developmental toxicology, teratology, validation metabolic activation system, cytochrome P-450, RA III
06	11		

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The goal of the project was to develop and validate the Frog Embryo Teratogenesis Assay-Xenopus (FETAX). FETAX is a 96 h whole embryo bioassay designed to rapidly assess the developmental toxicity of pure compounds or complex mixtures. The four specific objectives of this study are: 1) validate FETAX using direct-acting compounds of known mammalian developmental toxicity, 2) develop and validate an in vitro metabolic activation system using rat liver microsomes, 3) explore the use of three carrier solvents which will help solubilize non-polar compounds and, 4) develop an atlas of malformations which will assist users in performing FETAX. Five test compounds were selected for FETAX validation during the first year. Each compound was to be tested using 1-2 range finding tests and three definitive tests. Endpoints of FETAX are mortality, malformation and growth inhibition. Compounds selected for testing included three mammalian negatives and two strong positives. We have completedtesting on four of five compounds and testing is half completed for the fifth compound. FETAX correctly identified the developmental toxicity of four of the five substances

(over)

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified
22a NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia M. Miller	22b TELEPHONE (Include Area Code) 301/663-7325
	22c OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete

SECURITY CLASSIFICATION OF THIS PAGE

19. CONTINUED:

in the validation phase. The fifth compound tested correctly after the addition of the in vitro metabolic activation system. The development of the in vitro metabolic activation system using Aroclor 1254 induced rat liver microsomes is slightly ahead of schedule. Both Benzo(a)pyrene and Sodium Salicylate have responded as expected. The developmental toxicity of Benzo(a)pyrene is increased while developmental toxicity is essentially unchanged for Sodium Salicylate. Acetaminophen is presently giving the expected result but the data is somewhat variable. Initial experiments are being conducted on the other two compounds. The work on the carrier solvents has been carried out such that the initial dose-response curves for each of the three proposed carriers has been completed. Dimethyl Sulfoxide and Triethylene Glycol appear to be best suited for use as carriers but there will be situations where acetone may be required. We are now completing testing on the four compounds to be used in evaluating possible solvent interaction problems. Work has been completed on Trans-retinoic acid and 6-aminonicotinamide. When suitable dose-response curves have been generated for the remaining two compounds, interaction studies will commence. The "Atlas of Malformations" is being worked on. Micrographs made as part of the validation project are to be used in making the manual. We have already taken pictures for the first three chapters and are presently assembling them for review.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

NA Where copyrighted material is quoted, permission has been obtained to use such material.

NA Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

NA For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

NA In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PI Signatur[illegible] Date

TABLE OF CONTENTS

	Page
Front Cover	i
Report Documentation DD Form 1473	ii
Foreword	iv
Table of Contents	v
INTRODUCTION	1
Project Goals and Objectives	1
BACKGROUND	2
The Need to Screen for Teratogens	2
Development of FETAX as a Teratogenesis Screening Assay	2
In Vivo Bioactivation	4
In Vitro Bioactivation	4
Military Signifance	5
Methods of Procedure	7
1. Validation	7
2. Solvent Interaction Study	8
3. Metabolic Activation	8
4. Atlas of Malformations	9
TABLE 1	10
TABLE 2	11
TABLE 3	12
BODY	13
RESULTS AND CONCLUSIONS	13
1. Validation	13
Amaranth	13
Aspartame	22
5-Azacytidine	30
Methotrexate	40
d-Pseudephdrine	46
2. Solvent Interaction Study	55
Triethylene glycol	55
Acetone	64
Dimethyl sulfoxide	70
Trans-retinoic Acid	77
3. Metabolic Activation System	84
Acetaminophen	84
Acetazolamide	89
Benzo(a)pyrene	90
Dimethylnitrosoamine	96
Sodium Salicylate	99
LITERATURE REFERENCES	105
APPENDIX I Methods of Procedure	109

INTRODUCTION

Project Goal and Objectives

The goal of this research project is to develop and validate an in vitro bioassay for developmental toxicants using embryos of the South African clawed frog Xenopus laevis. The assay will allow rapid screening of pure compounds or complex mixtures as part of the hazard assessment process. The assay has been named the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) by its developer Dr. James Dumont (1) of Oak Ridge National Laboratories.

Four objectives have been established for the present project. These are:

1. Validation: FETAX will be tested using a number of pure test chemicals whose mammalian developmental toxicity has been previously established. The rationale for this work is to establish the predictive accuracy of FETAX and establish the false positive and negative rate. Chemicals that do not test as predicted will be retested using an in vitro metabolic activation system used in conjunction with FETAX. In this manner the validation work will help in the development of the assay. Attainment of this objective will also help in covalidation studies later that will be designed to show that FETAX is reproducible and reliable. Abnormal embryos generated during this phase of the testing will be photographed and the pictures used in preparing an "Atlas of Malformations" (objective #4).

2. Solvent Interaction Study: Some difficulty has been encountered in previous studies when non-polar test substances have been used. The use of dimethyl sulfoxide, acetone and triethylene glycol as carrier solvents is being evaluated in this study. The no observable effects concentrations of each of these solvents has already been determined but it is possible that these solvents may act synergistically or antagonistically with the test chemicals. It is an objective of this study to determine whether or not these type of interactions are occurring and how best to minimize them.

3. Validation of the Rat Liver Metabolic Activation System for FETAX: Aroclor 1254 induced rat liver microsomes are currently being used as an in vitro metabolic activation system for FETAX. Many proteratogens must be bioactivated before causing developmental effects while other compounds, such as nicotine, are rendered far less toxic. Since Xenopus embryos lack a functional metabolic activation system through the first four days of development, a substitute system must be exogenously provided. This system has been developed and used successfully. The objective in the present study is to validate the system in order to demonstrate that it increases the predictive accuracy of FETAX.

4. Atlas of Malformations: If FETAX is to provide repeatable and reliable data, it is imperative that a standardized methodology be provided to users. This guide is currently being developed through the auspices of the American Society for Testing and Materials. This new standard guide (See: Methodology Appendix) is mid-way through the development cycle. During its development

it became clear that new users were having difficulty recording which embryos were malformed and which stages of development they were dealing with. Photographs taken for the present project are being published in a book which will be provided to interested parties for the purpose training FETAX technicians. The repeatability and reliability of FETAX are very dependent on the quality of the technical help.

BACKGROUND

The Need to Screen for Teratogens

Approximately 50,000-70,000 chemicals are currently available in the marketplace with some 800 new chemicals released each year. Prior to their release into the environment, the safety of these chemicals must be firmly established. Because of the large numbers of chemicals to be tested and the even larger number of interactions possible when these chemicals are present in complex mixtures, *in vivo* assays employing mammals are not practical. The need for routine teratogenicity testing has led to the development of a number of *in vitro* teratogenesis assays that may prove useful in prioritizing compounds for further testing (R1-7). Several years ago, Dumont and co-workers (1) developed and used the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) and applied it to screening complex environmental mixtures as well as pure compounds. We propose to further develop FETAX and evaluate this assay using compounds of known mammalian developmental toxicity. Successful development and validation of this assay will make available to the scientific community a four-day screening test providing reliable developmental toxicity data.

Development of FETAX as a Teratogenesis Screening Assay

In its present form, FETAX meets most of the criteria set forth by Kimmel et al. (8) for the validation of *in vitro* teratogenesis assays (8, 9-11). Endpoints such as mortality, malformation, growth, development and motor impairment are easily quantifiable and capable of exhibiting a dose-response relationship with the establishment of narrow confidence limits. Since many of the stages of amphibian development are similar to mammalian development, the "developmental relevance" of FETAX is higher (8) than many of the other teratogenesis assays. At present FETAX has a metabolic activation system using uninduced rat liver microsomes, but improvements can be made. Additional validation using compounds of known mammalian developmental toxicity needs to be performed. The successful accomplishment of the specific aims listed below should make FETAX a fully developed and reliable teratogenesis screening assay.

The original work on developing an assay with *Xenopus* to detect environmental teratogens was performed in the laboratories of Greenhouse (7) and Dumont (12). Greenhouse used 48-hr exposures to military compounds (N-phenyl-a-naphthylamine and various hydrazines) to demonstrate toxic and teratogenic effects on developing embryos.

The early studies demonstrated that the *Xenopus* system can be used with a variety of chemicals and complex mixtures. The endpoints include: LC50 (mortality), EC50(malformation-teratogenesis), no observable effects concentration (NOEC), growth (both length and developmental stage obtained in

a given time period), motor behavior, pigmentation, and gross anatomy. The test chemical exposures are generally continuous for 96-hr (FETAX). Mortality and stage of development are checked at hours 24, 48, 72, and 96 hr, while the other endpoints are recorded only at 96 hr. Every 24 hr, fresh test compound and water is added (renewal). Data collection is simple as all observations are made with a dissection microscope. The data collected using FETAX are in harmony with the criteria for an in vitro teratogenesis screen given by Kimmel et al. (8). These include: good dose-response, adequate number of embryos, and easily defined endpoints.

FETAX Test Performance and Developmental Relevance

Dumont (unpublished) has validation data on 45 compounds with an approximate 85 percent correspondence to mammalian results. Sabourin (13-14) has completed testing of 32 compounds with 83 percent predictive accuracy. In our laboratory to date, we have tested 31 "direct-acting" compounds and found that 27 gave results in FETAX consistent with the mammalian literature for a predictive accuracy rating of 87%. In at least two cases (Isoniazid and D-Pseudoephedrine), we feel that metabolic activation is involved and that the mammalian literature has not properly taken this into account. Resolution of these problems in validation would increase the predictive accuracy significantly. However, we are bounded by the limits of the accuracy of the mammalian literature since funds to repeat mammalian studies are lacking.

Correlation between laboratories has been encouraging. For example, Courchesne and Bantle (9) found a teratogenic index for hydroxyurea of 4.3, whereas Sabourin recorded 4.5 for the same chemical. In some cases, however, a lack of correspondence is apparent. Even though 5-fluorouracil tested strongly positive for teratogenesis in the laboratories of Bantle and Sabourin, the teratogenic indices differed significantly. The reason for such discrepancies needs to be understood and the interlaboratory evaluation of the proposed study (year #2) will permit an evaluation of the interlaboratory variability. If the assay is to be used for purposes beyond screening, such as for ranking teratogenesis of test chemicals, then the importance of minimizing variability in quantitative measurements emerges.

Additional evidence which supports the use of FETAX as a viable teratogen screen concerns its performance compared with that of other potential test systems and its developmental relevance. Sabourin et al. (10) found the Xenopus test emerged as the assay of choice when compared to the planaria system (3) and the Hydra system (15). These results can be attributed to the numerous endpoints of the frog assay as well as its higher phylogenetic position. We have also found that endpoints in the Xenopus assay may demonstrate developmental relevance to those in mammalian systems. Sabourin and Carlton (unpublished) determined that the same stock of diphenylhydantoin caused pericardial edema as the primary endpoint in both the cultured whole rat embryo (16) and Xenopus embryos. We have also made this same observation. Dumont et al. (personal communication) similarly found that meclizine induced hydrocephalia in both frogs and mammals and that other teratogens produced similar abnormalities in both frogs and mammals. Sabourin (in preparation) has recently found that the total mammalian malformations (e.g., skeletal, visceral, nervous, etc.) caused by 17 teratogens were matched in Xenopus in 24/37 (65%) of the cases. Courchesne

and Bantle (9) reported that a number of genotoxic chemicals caused the same general types of malformations in both Xenopus and rodent embryos. Lastly, Dawson et al. (17) have developed a artificial medium (FETAX solution) and have carried out a preliminary validation using five compounds ranging from a nonteratogen to a strong teratogen.

In Vivo Bioactivation

A principal objective of this proposed study is to develop a means of dealing with proteratogenic substances in the FETAX system. Preliminary work, done independently in the laboratories of Dumont and Sabourin, indicated that a limited degree of basal P-450 activity and subsequently induced P-450 activity is present in the 96-hr embryo. This activity is not great enough to qualify FETAX as a metabolically competent system however. A significant increase in mortality or malformation between 72 and 96 hrs does indicate that bioactivation may be occurring later in development. We have observed this increase in mortality between 72 and 96 hrs using histamine. Wesolowski and Lyerla (18) have also reported that hexokinase and alcohol dehydrogenase activities do not appear until the fifth day of development in Xenopus.

In Vitro Bioactivation

We have developed an in vitro metabolic activation system (MAS) for FETAX by using Aroclor 1254 induced rat liver microsomes cocultured with embryos (19). Initial attempts to use commercially available Aroclor 1254 induced rat liver S-9 supernatant (Litton Bionetics) failed because of high S-9 toxicity. We then attempted to further purify microsomes from uninduced rat liver homogenate in order to reduce toxicity (20,21). We were successful in this approach and able to activate the proteratogen, cyclophosphamide into its embryotoxic and teratogenic form as measured by FETAX (19,20). We used cyclophosphamide in the development of uninduced rat liver microsome system for FETAX. Cyclophosphamide is one of the consensus compounds listed by Smith et al. (22) for use in validating in vitro teratogenesis assays. That cyclophosphamide requires metabolic activation for its teratogenicity has recently been shown using rat embryos cultured in vitro (23,24). We duplicated these experiments with FETAX to facilitate the development of our metabolic activation system. A wealth of literature exists on not only the metabolism of cyclophosphamide but also its mutagenic and teratogenic potential (See: Mirkes (25) for review). This makes it much easier to interpret results. Cyclophosphamide is easily soluble in water, eliminating the need for a carrier. Cyclophosphamide has now been adopted in our lab as a positive control to prove that each microsomal preparation is active.

Rat liver microsomes were prepared essentially by the method of Kitchin and Woods (20) and the method of preparation is presented in the Appendix (Section #1). Our chief contribution to the preparation and use of these microsomes in FETAX was the use of bovine serum albumin in reducing residual Aroclor to a level that does not affect experimental results and in the standardization of the amount of microsomal activity so that experiments could be repeated reliably (19). We have also established that microsomal activity lasts for five hrs at 24^{o}C.

After developing the in vitro MAS for FETAX, a validation program was initiated to prove that the system yields the expected results. Proteratogens, such as cyclophosphamide, should increase in developmental toxicity upon bioactivation while others should be reduced. FETAX results using direct-acting developmental toxicants should not change upon addition of the in vitro MAS. Besides cyclophosphamide, we have also tested the proteratogens 2-acetylaminofluorene, rifampicin and benzo(a)pyrene in FETAX and obtained clear evidence of bioactivation for each (26). Both nicotine and cytochalasin D are examples of compounds that are inactivated by an in vitro MAS and we obtained similar results in FETAX (26,27). Lastly, we have already used $ZnSO_4$ as an example of a compound that should be unaffected by the MAS and obtained the expected results in FETAX (26). With Zn we had questioned whether or not rat liver metallothioneins would bind to the zinc and reduce its toxicity. This did not turn out to be a problem. Lastly, we have tested dilantin as an example of a compound that has both direct-acting and possibly developmentally toxic intermediates (28). Using specific inhibitors of P-450 enzymes, we were able to show that dilantin caused developmental toxicity in its parent form and that there was evidence for toxic intermediate but that the primary metabolites were not as toxic as the parent compound.

We did try to use Xenopus hepatocytes in a co-culture with Xenopus embryos as an alternate in vitro MAS. An advantage of using cultured hepatocytes as a bioactivating system would be the continuous production of the active metabolite as opposed to the use of rat liver microsomes where activation is limited to approximately 5 hrs (although fresh additions are made daily). However, Xenopus hepatocytes do not have as much inducible cytochrome P-450 as do rat liver microsomes and rat hepatocytes cannot be used because of the tonicity differences between amphibian and mammalian cells. Cell culture is far more expensive and technically demanding than the use of rat liver microsomes. Further, there are problems in plating an exact number of hepatocytes so that the generation of teratogen from proteratogen is repeatable. It must be remembered that every in vitro metabolic activation system has advantages and disadvantages and that no universally accepted system now exists that duplicates human metabolism.

At present, we feel that the use of Aroclor 1254 induced rat liver microsomes as an in vitro metabolic activation system for FETAX offers great promise. Additional validation compounds must be tested to ensure that there are no surprises that may affect testing results. However, assuming that there are none we feel that the MAS will greatly enhance the predictive accuracy of FETAX and extend the utility of the assay.

Military Significance

As stated in the USAMRDC Broad Agency Announcement, the proposed bioassay would fall under the area of Section C. Army systems hazards. This would include both health hazards of military material (part 2) and military environmental quality (part 4). Virtually any compound or mixture that is or can be made water soluble can be tested by FETAX for developmental effects.

FETAX is an alternative which offers some short and long-term solutions to current problems associated with developmental hazard evaluation. The

problems are: increasing regulatory requirements, test cost, test duration, and the reduction in the use of mammals in research. Existing in vivo mammalian test systems are effective for the testing of drugs and cosmetics but are too lengthy for the screening of water samples and other complex mixtures. In addition, the number of chemicals in production which require testing is increasing at a faster rate than is feasible to handle with in vivo test systems. Animal rights groups have increased pressure to slow down or halt the use of mammalian in vivo test systems. These events have produced a climate which favors the use of alternative test systems for eventual refinement, reduction, and replacement of in vivo assays. In a study conducted for NIH, the National Academy of Sciences recently concluded that the development and use of lower form models for toxicity testing should be emphasized (29). Tighter restrictions will limit the use of in vivo assays and increase costs associated with their performance. The Office of Technology Assessment for the United States Congress has recently completed an assessment of the use of animals in testing, research, and education (30). Congressional options emanating from this study include support for the further development and use of in vitro test systems when appropriate.

Cost-effective, yet developmentally relevant, tests are urgently needed to allow the detection of developmental toxicants in the environment and to assess the developmental toxicity of pure compounds and drugs. FETAX offers the advantage of detecting the toxicity and teratogenicity of complex environmental mixtures where the individual action of each component may be known but the combined action cannot be predicted.

Because of its position on the phylogenetic scale, Xenopus provides the highest degree of developmentally relevant endpoints compared to other in vitro teratogenesis assays such as cell culture, planarian, fruitfly, and Hydra systems. The costs of performing the above mentioned tests are similar, except for cell culture which is higher. Xenopus can be considered a high connectivity model due to the amount of biological information available on this species (29). There is a good chance that observations made on this species can be connected to data from other organisms, including man. Xenopus has been a standard lab animal for numerous developmental studies and the Xenopus oocyte is a commonly used biomedical model. Consequently, much is known about the frog's morphology, physiology, and biochemistry. This cannot be stated for many of the alternative systems. Xenopus can be bred throughout the year and provides numerous offspring in order to facilitate statistical analysis. Because development is external, the speed of data collection is enhanced. The lack of a placental relationship is a disadvantage, but this problem is shared with other emerging in vitro teratogenesis assays.

The research plan we are following is designed to improve and evaluate the FETAX system and to critically test the assumption that FETAX can adequately detect mammalian teratogens. Successful efforts in developing a reliable metabolic activation assay for Xenopus embryos may provide a viable adjunct to in vivo mammalian assays. The need for representative metabolism is considered a requirement for adequate use of in vitro systems in screening potential teratogens (8). A bioactivation system will improve the predictive capability as well as enhance the usefulness of the FETAX test. The assay at present appears capable of detecting over 85% of the teratogens from a moderate group of chemicals (including proteratogens)

with few false positives. Since evidence indicates that metabolic activation probably plays a role in teratogenesis (30, 31), much the same as in carcinogenesis, the proposed efforts are indeed significant.

Methods of Procedure

1. Validation

We predict that FETAX will perform at the 85% level in terms of its sensitivity and specificity when tested with compounds whose mammalian and human developmental toxicity are known. Controversy does exist about how best to conduct validation studies in this area. The list of compounds published by Smith et al. (22) in 1983 has merit but it has been criticized by many as being too small and weighted toward positives likely to cause teratogenic insult in virtually all species. Additionally, some compounds were listed based on experiments that used routes of administration such as inhalation We feel this list has been much maligned but that it still as a reasonable starting point for a validation study so long as its limitations are accounted for. Marshall Johnson (32) feels that in vitro teratogenesis testing data should be compared to segment II studies performed with rats. This idea has merit but many of these studies have been performed for the FDA and the results are not made public. Nigel Brown (33) has recommended that testing be done "in the blind" and we have done this for the validation compounds listed in Table 1. Lastly, some believe that the only proper method of comparing the predictive accuracy of validation studies is to perform mammalian tests in conjunction with in vitro tests. Obviously this is the method of choice were it not for the ruinous cost associated with mammalian tests requiring a dose-response effect. In selecting compounds for testing we consider the Smith et al. list, Shepard's "Catalog of Teratogenic Agents" (34), Segment II data gleaned from the literature and any other studies published by good researchers in the field of mammalian teratology. This is a compromise approach but one which we feel has been successful to date. When a compound fails to perform as expected we try the assay again using the rat liver MAS to see if bioactivation or deactivation affected results.

No rules exist as to how many compounds need to be tested before an assay is "acceptable" to regulatory agencies. The Chernoff mammalian teratogenesis screen has been tested with > 150 compounds (See:Teratogenesis, Carcinogenesis and Mutagenesis Vol. 7(1)). FETAX has undergone validation with 90 compounds although the results from 40 compounds have not yet been published by Dumont because he has left the field (J.N. Dumont personal communication). Dumont did not use the renewal procedure and left the jelly coats on the eggs. These differences in procedure from the ASTM guide necessitate additional testing to further validate the test using the same methodology.

Any substantive modifications of this procedure made at future ASTM meetings will be adopted although it is unlikely that this will occur based on the results of the first subcommittee ballot.

Successful validation of FETAX using the compounds proposed will allow its use as an in vitro teratogenesis screening assay. Final acceptance of the assay will depend on a high degree of predictability probably at the 85% level. As of this writing FETAX is now performing at this level.

Solvent Interaction Study

The solvents and test compounds for this study are listed in Table 2. To date Triethylene Glycol, Acetone and Dimethyl Sulfoxide are among the best candidates to assist in solubilizing nonpolar compounds so that they can be tested. Triethylene Glycol is the least toxic and teratogenic but it is not as good a solvent as the other two. Acetone is a good solvent but its TI is slightly high. Dimethyl Sulfoxide seems to be the best solvent available. In previous tests, concentrations of solvent at the NOEC had no effect on the activity of the in vitro metabolic activation system employing rat liver microsomes. The four tests compounds listed in Table 2 will be used to show how much the inclusion of solvent to the FETAX protocol affects test results. Financial support for finding the 96-hr LC50, 96-hr EC50 (malformation) and MCIG for each compound is being provided from another source.

Dose-Response curves for mortality and malformation will be derived for each test compound and solvent. Experiments will be conducted by starting at the 96-hr LC25 and EC25 (malformation) for each test compound. One series of experiments will be performed at the highest no observable effect concentration (NOEC) for each solvent. A second series of experiments will be at the 96-hr LC25 and EC25 (malformation) for each solvent and test compound. The appropriate control will be the test compound in water only. It may prove difficult to get good numbers for the insoluble test compounds so it may be necessary to compare results between the solvent NOEC and the solvent 96-hr LC25 and EC25 (malformation). If there is a positive interaction between the solvent and test compound, then the effects observed will be greater than simple additive effects and if there is a negative interaction, then mortality, malformation and growth effects will be less. For each experiment there will be four dishes of 25 embryos each in FETAX solution as controls. Analysis of data and statistics will be as previously published for FETAX interaction studies (35).

3. Metabolic activation

We are trying to answer the question of whether we can develop and validate an in vitro metabolic activation system for FETAX using Aroclor 1254 induced rat liver microsomes that closely simulates mammalian metabolism. Although it will not be the same type of metabolic activation system as found in humans, it should be close enough to detect most mammalian and human pro-teratogens.

Section 1 of the Appendix details our method of making microsomes and how we standardize the units of P-450 activity. Now that we have developed this method, we are actively engaged in validating the use of these microsomes and fine tuning the procedure for maximum efficiency. We have selected an additional set of 5 compounds to test in this study. Two compounds require activation for a developmentally toxic effect, two are direct-acting teratogens and a third compound appears to be a negative that is not activated (Table 3). Our plan is to perform two experiments on each compound with and without in vitro metabolic activation system. Because

of the complexity of these experiments only 20 embryos per dish will be used instead of the standard 25. Dose-response curves for mortality and malformation will be compared as well as teratogenicity indices (TIs). The relative changes in the 96-hr LC and EC50s will indicate whether any bioactivation or inactivation has occurred. Similarly growth curves can be compared to determine whether metabolic activation was required. Compounds listed on Table 1 will tested using the in vitro MAS if they do not performed as expected in the standard FETAX assay. In this case tests will be run only at the direct-acting 96-hr LC and EC50 concentrations. Increases or decreases from the 50% median effect levels (mortality and malformation) will serve as an indication as to whether the microsome-treated embryos differ from controls due to metabolism.

4. Atlas of Malformation

Since we have already received the Wild photomacroscope needed to take pictures of the malformed embryos in this study, we are in an excellent position to develop this atlas as part of this project. The publications department of Oklahoma State University has estimated that a suitable atlas would cost $7,000 for 750 copies. The cost reflects the large number (100) of photographs needed to illustrate the various malformations. The labor in taking the photographs and preparing the atlas is part of the current contract. The manual will be provided to the Army, Army contractors and other individuals gratis until the supply is exhausted. Postage and handling charges would be the only cost borne by the new user. No royalties will accrue to the PI.

The manual will cover mating the frogs, staging of embryos, diseases of adult frogs and their remedies and, lastly, all the major malformations. Particular emphasis will be placed and judging slight malformations as this affects the scoring of the malformation endpoint the greatest. We have saved all the embryos from each experiment and have gone through them looking for each different type of malformation. Once we get a complete series (ex. all possible eye malformations) we begin photographing them with a Wild photomacroscope. Color negatives are made and all of the negatives are first printed in black and white. We then arrange the best micrographs and select which need to be presented in color and which can be presented in black and white. These micrographs are then pasted up and the legends are added. Dr. James Dumont and our COR, Dr. Robert Finch then comment on the chapter. After this the chapter is revised and then sent to the publications department of Oklahoma State University for processing and printing.

Table 1. List of Proposed Test Compounds for FETAX Validation.

Compound	CAS #	MAS	Teratogen*	Solu-bility ***	Range Finder/ Definitive 1 LC50 EC50 TI**	Definitive 2 LC50 EC50 TI	Definitive 3 LC50 EC50 TI
Validation Compounds							
Amaranth **** (mg/ml)	915-67-3	N	-	1	2.67 3.5 0.8 (MCIG 3.0)	3.68 3.1 1.2 (MCIG>4.0)	3.81 3.91 0.97 (MCIG 3.75)
Aspartame (mg/ml)	22839-47-0	N	-	3	>8.0 >8.0 NA (MCIG 3)	>10.0>10.0 NA (MCIG NA)	13.9 13.1 1.1 (MCIG 7)
5-Azacytidine (mg/ml)	320-67-2	N	+	1	0.59 0.014 42 (MCIG 0.04)	0.62>0.05 NA	0.6 0.07 9 (MCIG 0.1)
Methotrexate****	59-05-2	N	+	1	R 0.79 0.02 43 (MCIG 0.05)	0.51 0.02 23 (MCIG 0.02)	
d-Pseudoephedrine HCl (mg/ml)	345-78-8	N	-	2	0.44 0.26 1.7 (MCIG 0.2)	0.42 0.23 1.8 (MCIG 0.2)	0.39 0.21 1.9 (MCIG 0.15)
d-Pseudoephedrine HCl (mg/ml)*****	345-78-8	N	-	2	ca.0.43 0.21 NA	0.43 0.23 NA	
		Y	-	2	>0.43 >.43 NA	>0.43 >.43 NA	

* + definite mammalian teratogen, - nonteratogen, V(+) variable positive- substance has been tested in a number of mammalian species and is generally positive, V(-) variable negative.
** Range finder is listed if the first definitive test has not yet been done. The numbers terminate in an "R" for range finder. If there is no "R" suffix it is a definitive.
*** 1-freely, 2- good, 3-fair, 4-poor, 5-insoluble.
**** Listed in Smith et al., Teratogenesis, Carcinogenesis and Mutagenesis 3:461-480, 1983.
***** A limited test with rat liver microsomes was conducted with psuedoephedrine. Only a limited number of concentrations were used. In the presence of microsomes, there was no observeable toxicity or teratogenicity.

Table 2. List of Proposed Test Compounds for Determining Possible Solvent Interaction Effects.

Compound	CAS #	MAS	Teratogen*	Solubility ***	Range Finder/ Definitive 1 LC50 EC50 TI**	Definitive 2 LC50 EC50 TI	Definitive 3 LC50 EC50 TI
Solvents							
Triethylene Glycol (% v/v)	112-27-6	N	-	1	2.4 2.0 1.2 (MCIG 1.8)	2.75 2.4 1.1 (MCIG 1.8)	2.19 2.05 1.07 (MCIG 1.7)
Acetone (% v/v)	67-64-1	N	-	2	2.16 1.4 1.6 (MCIG 1.25)	2.49 1.4 1.8 (MCIG 1.5)	1.92 1.06 1.83 (MCIG 1.0)
Dimethyl sulfoxide (% v/v)	67-68-5	N	V(-)	1	1.81 1.4 1.3 (MCIG 1.25)	1.77 1.29 1.4 (MCIG 1.7)	1.86 1.24 1.5 (MCIG 1.2)
Compounds used in Solvent Interaction Study							
trans-Retinoic acid (ug/ml) ****	302-79-4	N	+	5	0.246 .024 10 (MCIG 0.06)	0.50 0.044 11 (MCIG 0.08)	
Me-Mercury Chloride ****	115-09-3	N	+	4			
Trichloroethylene (% v/v) ****	79-01-6	N	-	4	R 0.06 0.015 3.7		
6-aminonicotinomide ****	329-89-5	N	+	1	Values already determined and in Press in "Drug and Chemical Toxicology"		

* + definite mammalian teratogen, - nonteratogen, V(+) variable positive- substance has been tested in a number of mammalian species and is generally positive, V(-) variable negative.
** Range finder is listed if the first definitive test has not yet been done. The numbers terminate in an "R" for range finder. If there is no "R" suffix it is a definitive.
*** 1-freely, 2- good, 3-fair, 4-poor, 5-insoluble.
**** Listed in Smith et al., Teratogenesis, Carcinogenesis and Mutagenesis 3:461-480, 1983.

Table 3. List of Proposed Test Compounds for FETAX Metabolic Activation System Validation.

Compound	CAS #	MAS	Teratogen*	Solubility ***		Range Finder/ Definitive 2			Definitive 3		
						LC50	EC50	TI**	LC50	EC50	TI
Validation Compounds for Metabolic Activation System											
Acetaminophen (mg/ml)	103-90-2	?	V(-)	2	NO MAS	0.15	0.13	1.1	0.19 (MCIG 0.1)	0.13 (MCIG <.12)	1.5	>3.0 (MCIG 0.15)******	0.1	>3.0
Acetazolamide **** (mg/ml)	59-66-5	N	V(+)	3	R NO MAS	>0.1	>0.1 *****							
Benzo(a)pyrene (ug/ml)	50-38-2	Y	+	4	MAS	>10.0	0.2	>5	>10.0	1.5	>6.7	>10.0	1.8	>5.6
					NO MAS	>10.0	10.0	>1	>12.0	10.0	>1.2	>10.0	10.0	>1.0
Dimethylnitrosamine (mg/ml)	62-75-9	Y	+	2	NO MAS	3.5	2.3	1.6						
Sodium Salicylate (mg/ml)	54-21-7	N	V+	1	MAS	2.2	1.4	1.6	2.3	1.5	1.5			
					NO MAS	2.34	1.7	1.4	2.3	1.23	1.87			

* + definite mammalian teratogen, - nonteratogen, V(+) variable positive- substance has been tested in a number of mammalian species and is generally positive, V(-) variable negative.
** Range finder is listed if the first definitive test has not yet been done. The numbers terminate in an "R" for range finder. If there is no "R" suffix it is a definitive.
*** 1-freely, 2- good, 3-fair, 4-poor, 5-insoluble.
**** Listed in Smith et al., Teratogenesis, Carcinogenesis and Mutagenesis 3:461-480, 1983.
***** Limit of solubility in 1% DMSO.
****** with penicillin-streptomycin.

Results

1. Validation

Table 1 shows the validation data collected to date. When a compound is tested in FETAX, range finding experiments are first conducted in order to determine the best concentrations of toxicant to use in establishing mortality and malformation dose-response curves. The results of these tests are not present in Table 1. After the proper concentration ranges are selected, then at least three definitive tests are performed in the blind. In practice, Mr. Doug Fort labeled the compounds A, B, C, etc. and handed them to the technician (Mrs. Shirley Bush) who then conducted the assay. She worked under high hazard containment at all times as she did not know the identity of the test compound. It must be remembered that the dose-response curves presented only show the data points (50 embryos per data point) used in constructing the dose-response curve. In practice there were many additional concentrations tested in each experiment.

The compounds selected for testing were Amaranth, Aspartame, 5-Azacytidine, Methotrexate and d-Psuedoephedrine. Based on the mammalian literature, 5-Azacytidine and Methotrexate were thought to be positives while the other compounds were thought to be negatives. In the case of d-Psuedoephedrine, the in vitro metabolic activation system was used because results were not as anticipated. This data and its comparison to controls is listed in the last row of Table 1.

Amaranth: Amaranth (Red food dye #2) is a compound listed by Smith et al. (22). It has generally been shown to be a nonteratogen in mammalian and human studies. Shepard (34) lists 4 studies on Amaranth with only one report dissenting from the conclusion that Amaranth is a not a developmental toxicant. Amaranth is compound that has been independently tested in FETAX before by both Sabourin and Faulk (14) and James Dumont (Oak Ridge National Labs-personal communication). They found that Amaranth was a negative as well. We repeated their work with this compound in order to estimate the reliability and repeatability of FETAX and to detect any co-validation problems early on with FETAX. It must be remembered that both Sabourin and Faulk and Dumont both performed FETAX slightly differently than the ASTM Guide.

Table 1 shows that Amaranth is clearly not a developmental toxicant. The average TI was 0.99 and the MCIG (minimum concentration that statistically inhibits growth at P=0.05) averaged 3.58. This was even greater than the average LC50 of 3.39. Development toxicants generally inhibit growth at concentrations far less than the 96-hr LC50. Figures 1, 3 and 5 show the dose-response curves for all three definitive experiments. While not superimposable, it is clear that the results pointed to the same conclusion. Figures 2, 4 and 6 show that there is little effect on growth regardless of the concentration used. Plates 1A and 1B show micrographs of larvae exposed to Amaranth continuously for 96 hr. Plate 1 is blurred slightly because this micrograph was taken before we got a vibration mount for our photomacroscope. Nonetheless, the micrographs show that only concentrations well above the 96-hr LC50 cause malformation. Strong teratogens affect development at concentrations far below the 96-hr LC50. Even at high Amaranth concentrations, the malformations are not severe. We

conclude that Amaranth was not a developmental toxicant in FETAX and this agrees with the vast majority of mammalian literature.

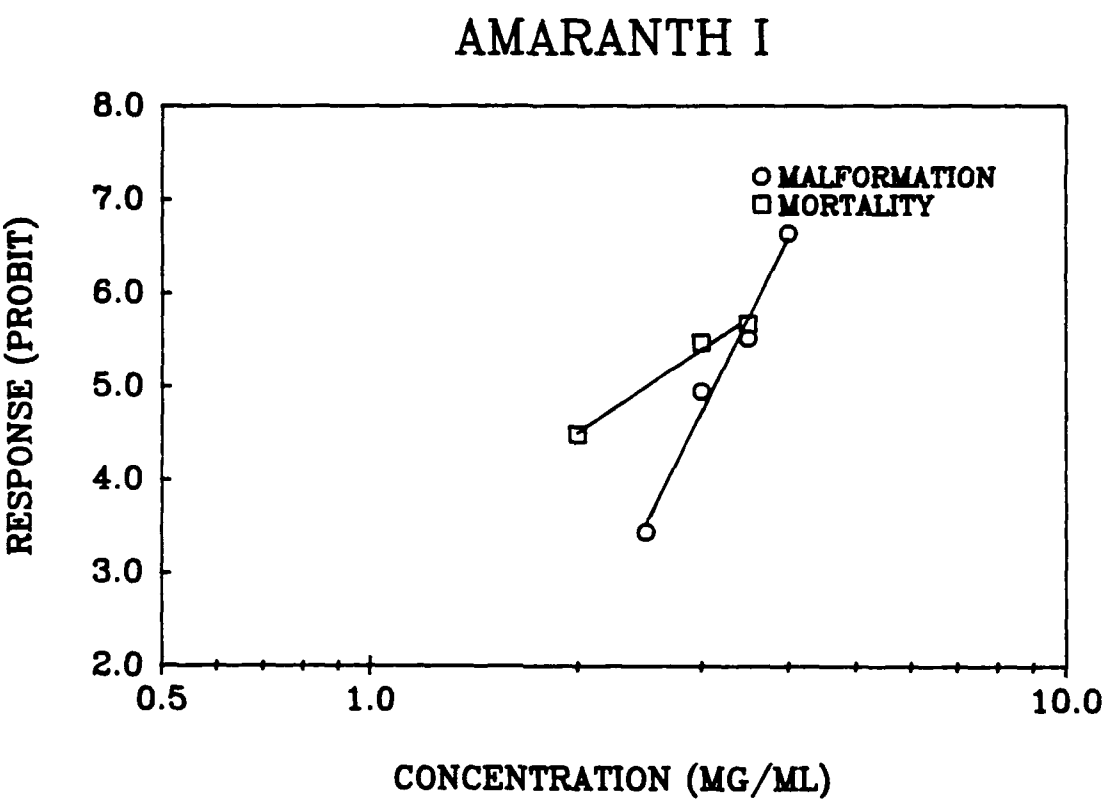

Figure 1. 96-h Mortality and Malformation Dose-Response Curves for Amaranth, Defintitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

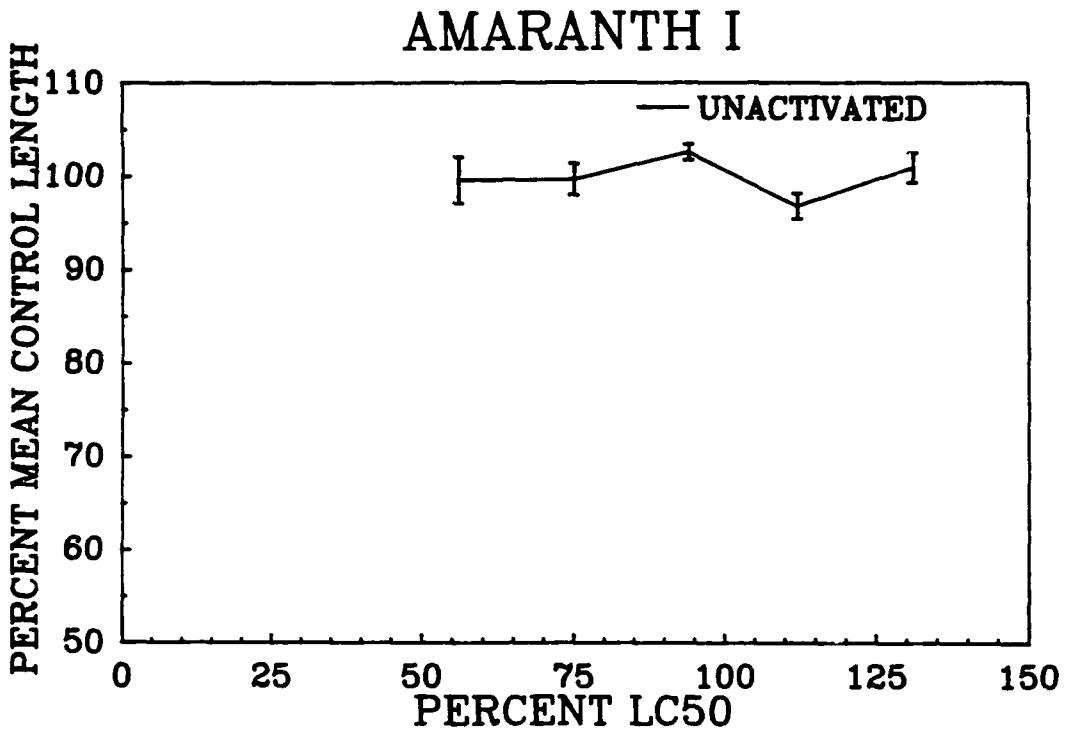

Figure 2. 96-h Growth Dose-Response Curve for Amaranth, Defintitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

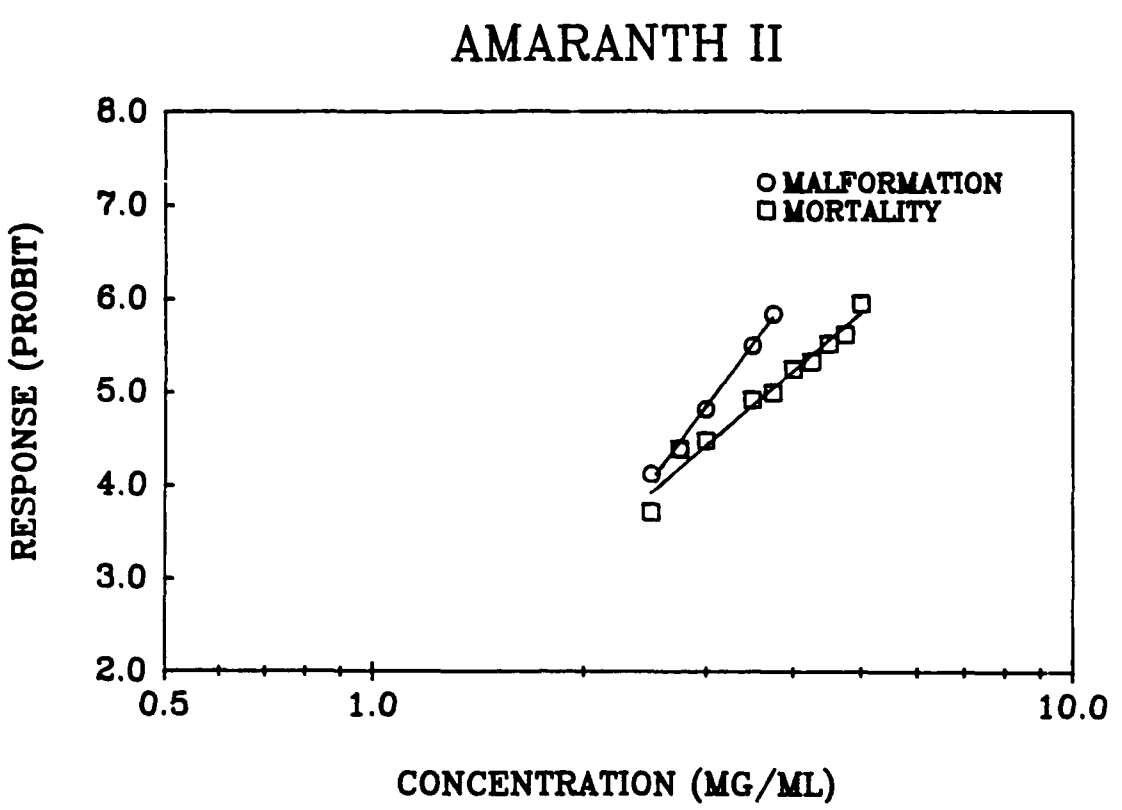

Figure 3. 96-h Mortality and Malformation Dose-Response Curves for Amaranth, Defintitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

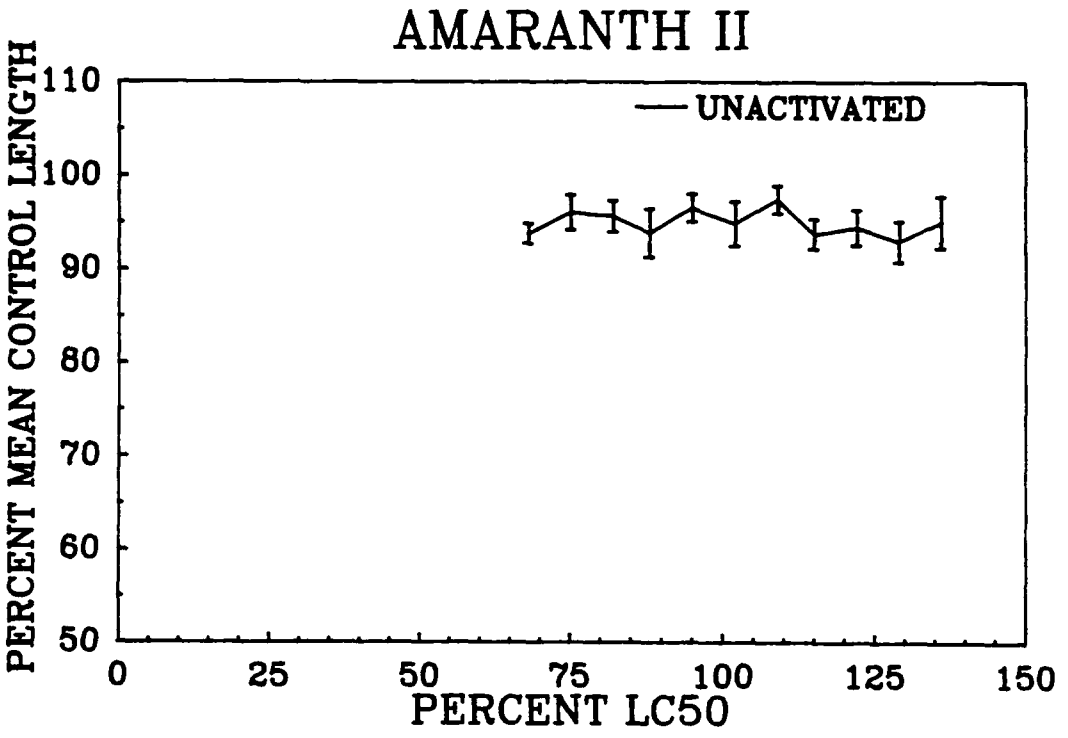

Figure 4. 96-h Growth Dose-Response Curve for Amaranth, Defintitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

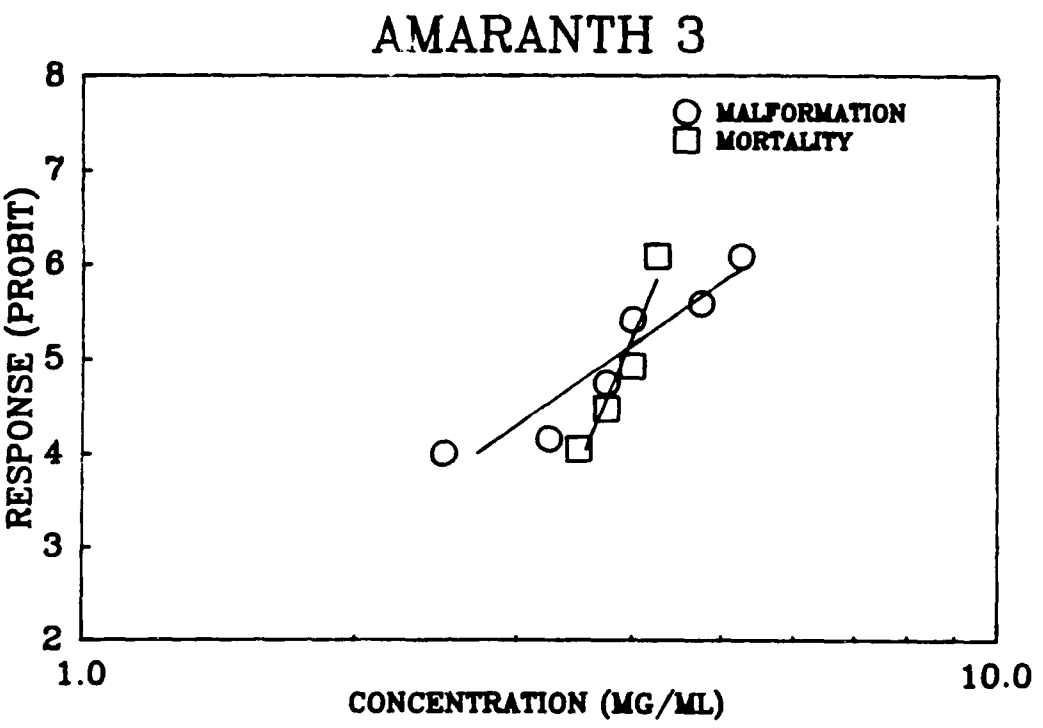

Figure 5. 96-h Mortality and Malformation Dose-Response Curves for Amaranth, Defintitive Test #3. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

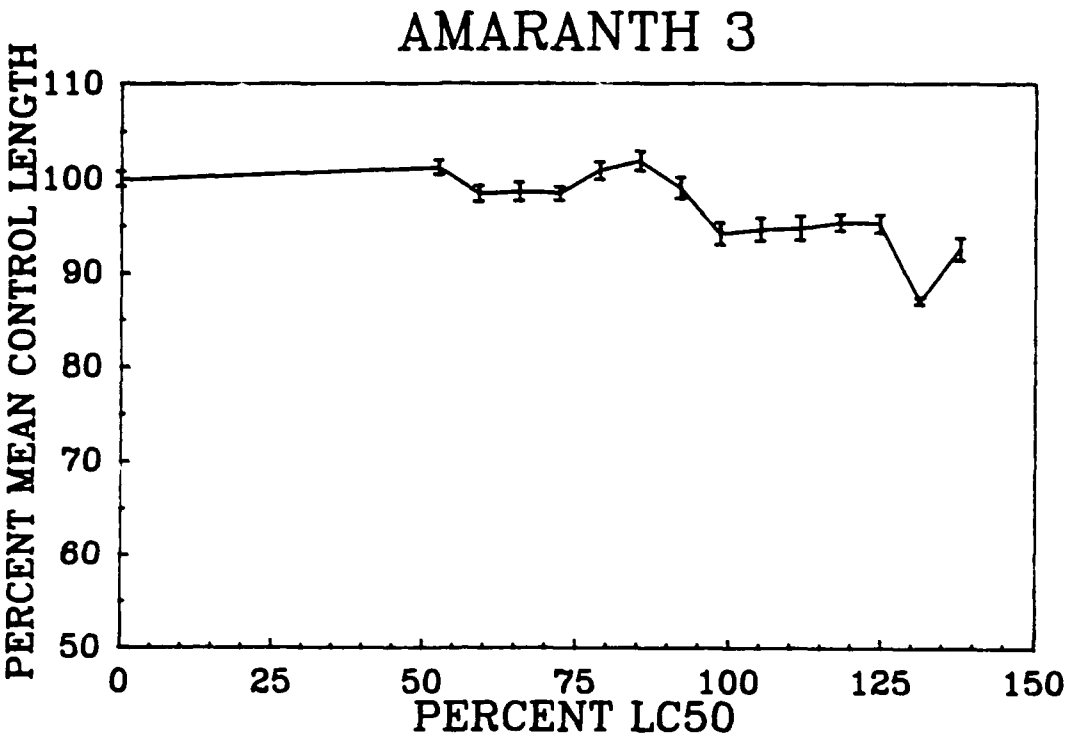

Figure 6. 96-h Growth Dose-Response Curve for Amaranth, Defintitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

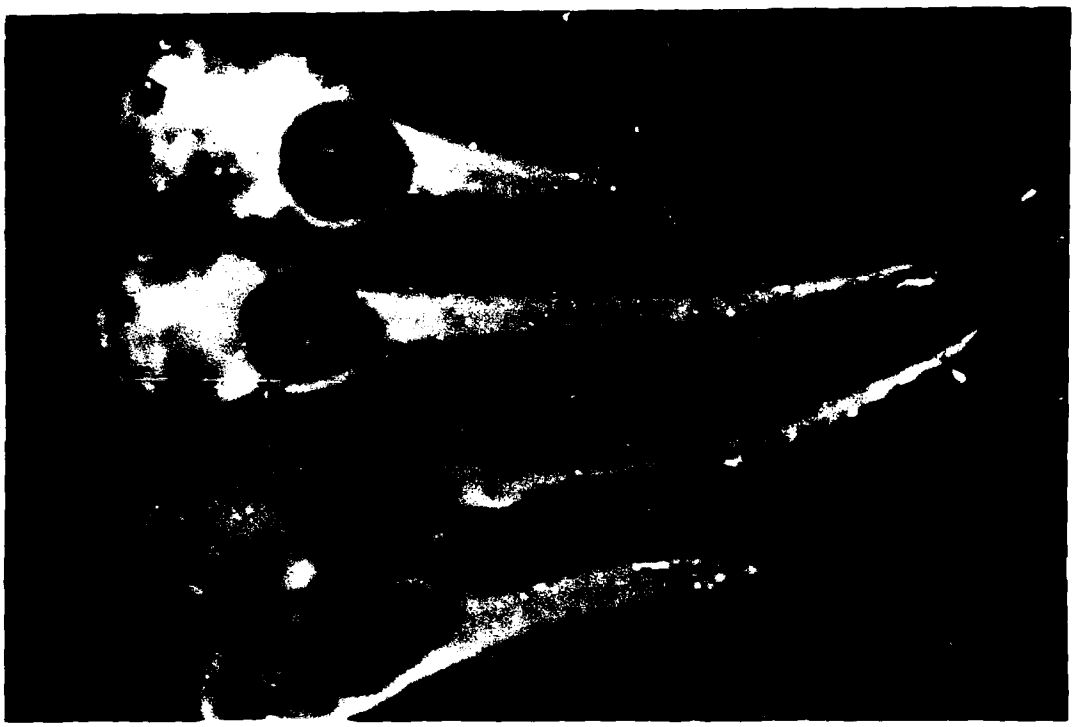

Plate 1A. Effects of Different Concentrations of Amaranth on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Ventral view presented to show effect on gut coiling. From top to bottom: control, 2.5 mg/ml, 4.25 mg/ml, 5 mg/ml.

Plate 1B. Effects of Different Concentrations of Amaranth on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 2.5 mg/ml, 4.25 mg/ml, 5 mg/ml.

Aspartame: (Nutrasweet- an artificial sweetner) is another water soluble compound that is not a developmental toxicant (34). It is not on the Smith list. Table 1 shows that is was essentially nontoxic even at concentrations approaching its limit of solubility in water. In the third definitive experiment, we were able to derive a TI of 1.1 for Aspartame by extrapolating the data to the 50% effect levels. This result is compatible with the shapes of the mortality and malformation dose-response curves seen in Figures 7, 9 and 11. The slope of the dose-response curve is very gradual and the two curves are very close to one another. All three growth-inhibition curves (Figs. 8, 10 and 12) are consistent and suggest that while growth inhibition with Aspartame is greater than Amaranth, it is still not a serious growth inhibitor. Plate 2B shows that Aspartame does cause more severe malformations than Amaranth. However, it does take high concentrations around 7-8 mg/ml to cause these malformations. When they occur, virtually all organ systems of the body are affected with equal frequency. Rupture of the eye is common at the higher concentrations. Cardiac and gut malformations are common over wide concentration ranges but curvature of the spine seems to occur only at the highest concentrations. Aspartame is still a negative because of its low TI and its limited effect on development but it is more embryotoxic, teratogenic and growth inhibiting than Amaranth.

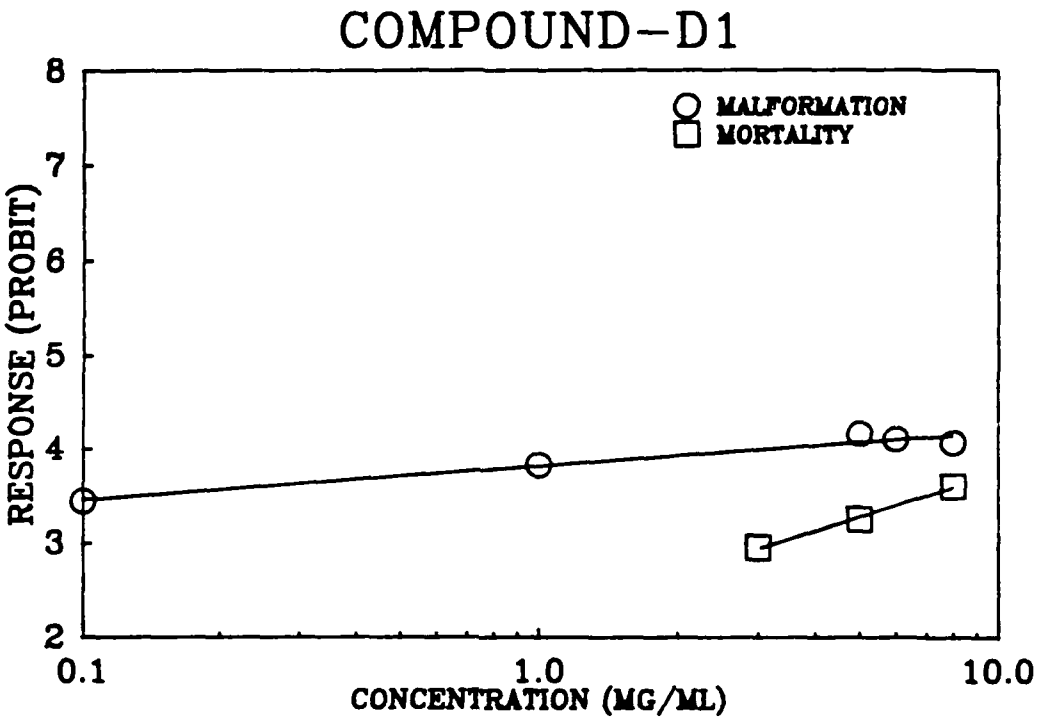

Figure 7. 96-h Mortality and Malformation Dose-Response Curves for Aspartame Defintitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

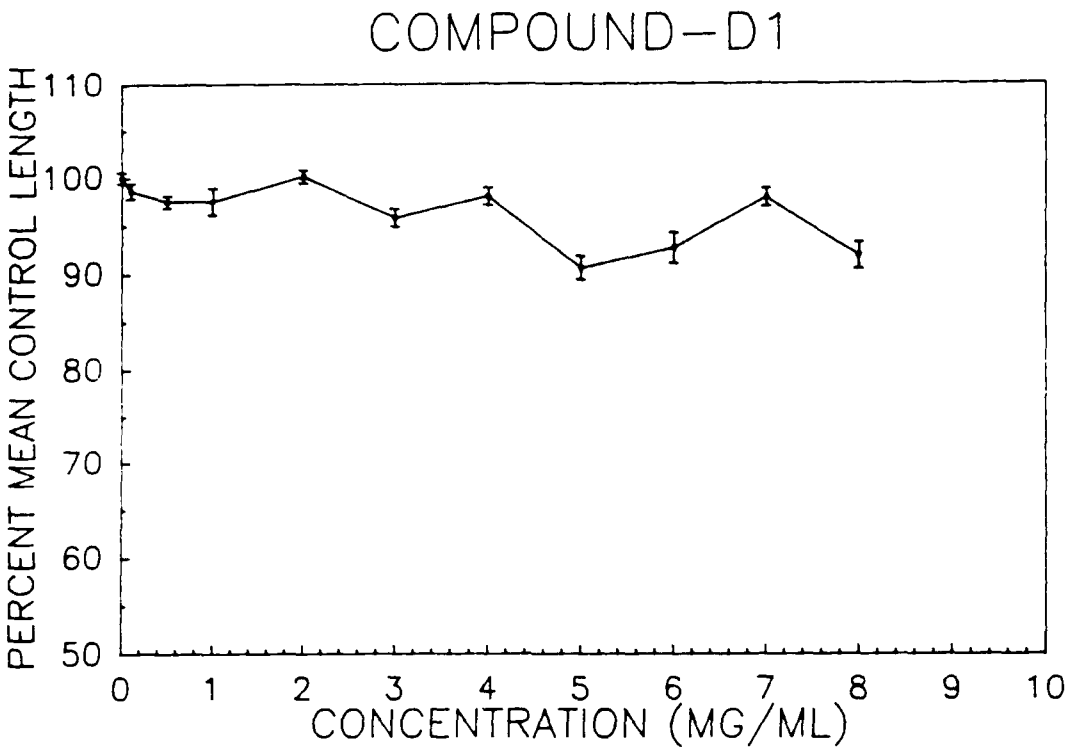

Figure 8. 96-h Growth Dose-Response Curve for Aspartame Defintitive Test #1. The plot shows the % of control versus the % of the 96-h *LC50*. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

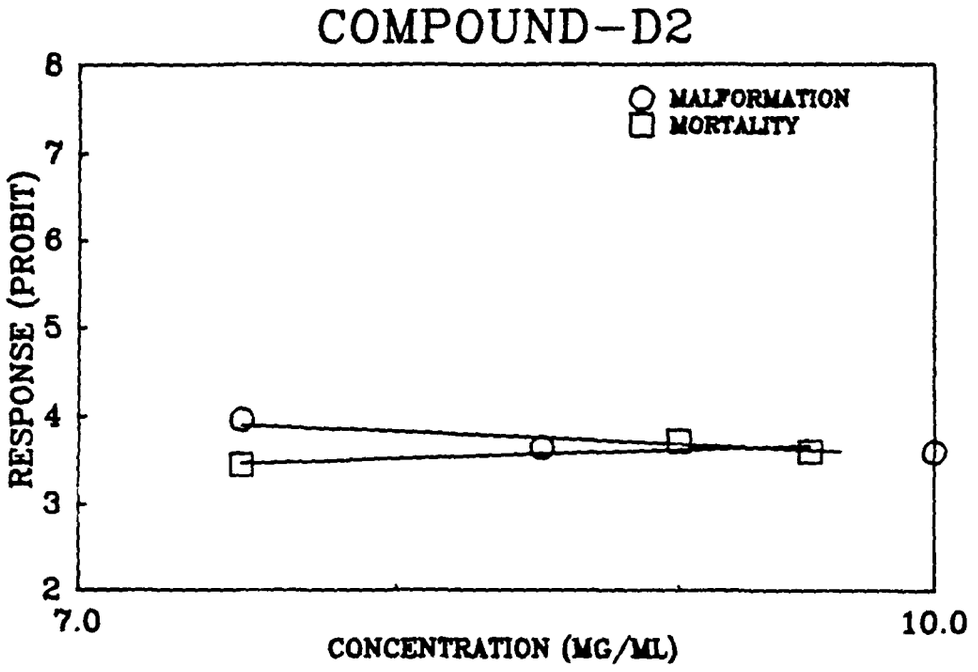

Figure 9. 96-h Mortality and Malformation Dose-Response Curves for Aspartame Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

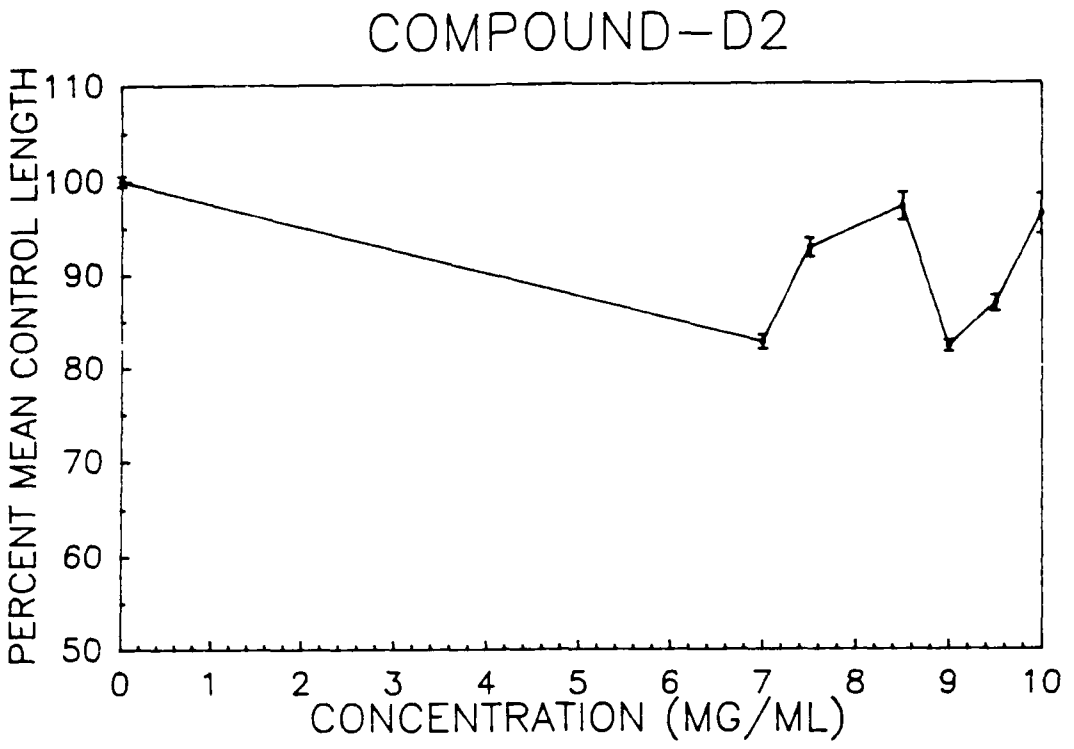

Figure 10. 96-h Growth Dose-Response Curve for Aspartame Defintitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

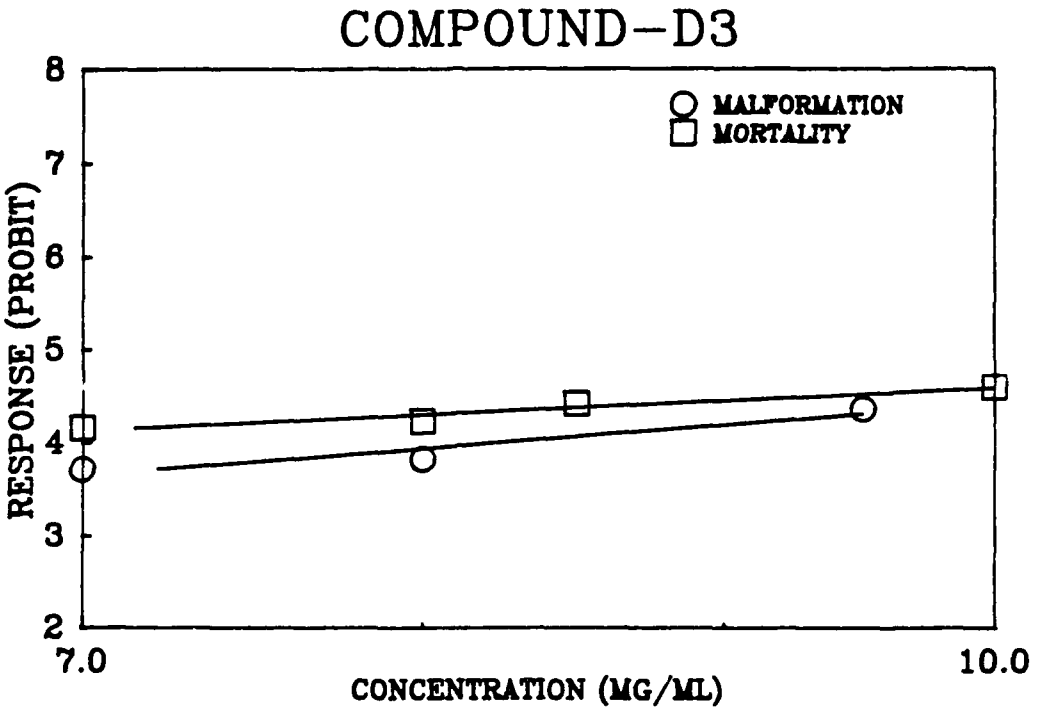

Figure 11. 96-h Mortality and Malformation Dose-Response Curves for Aspartame Defintitive Test #3. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

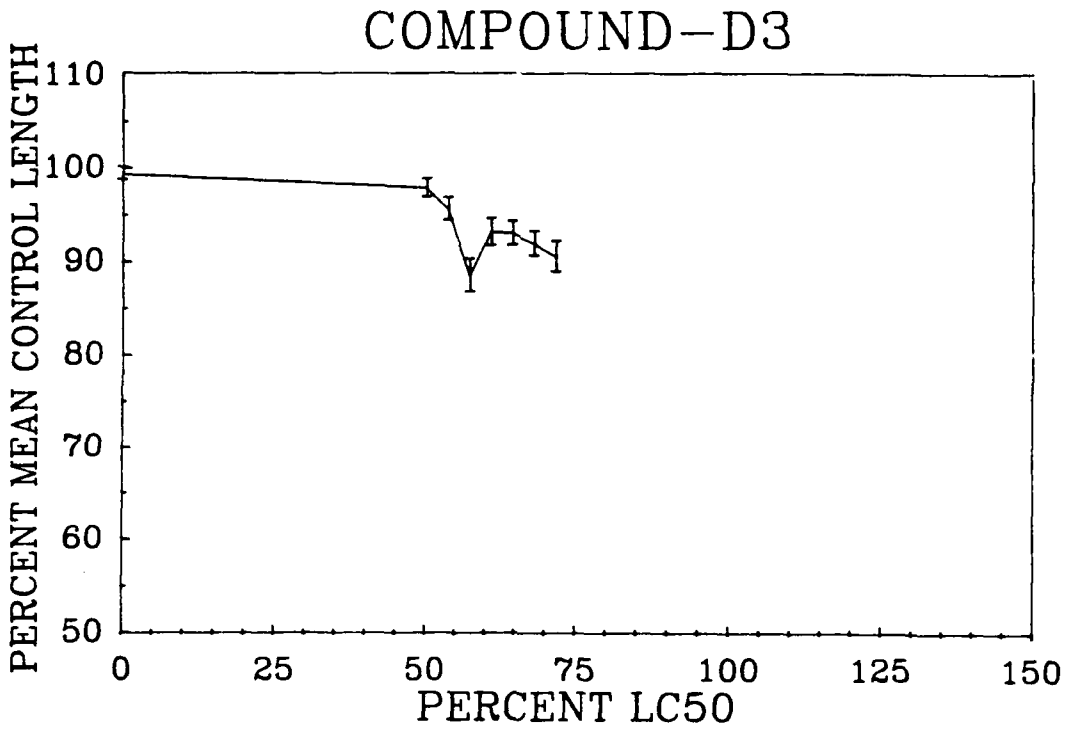

Figure 12. 96-h Growth Dose-Response Curve for Aspartame Defintitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

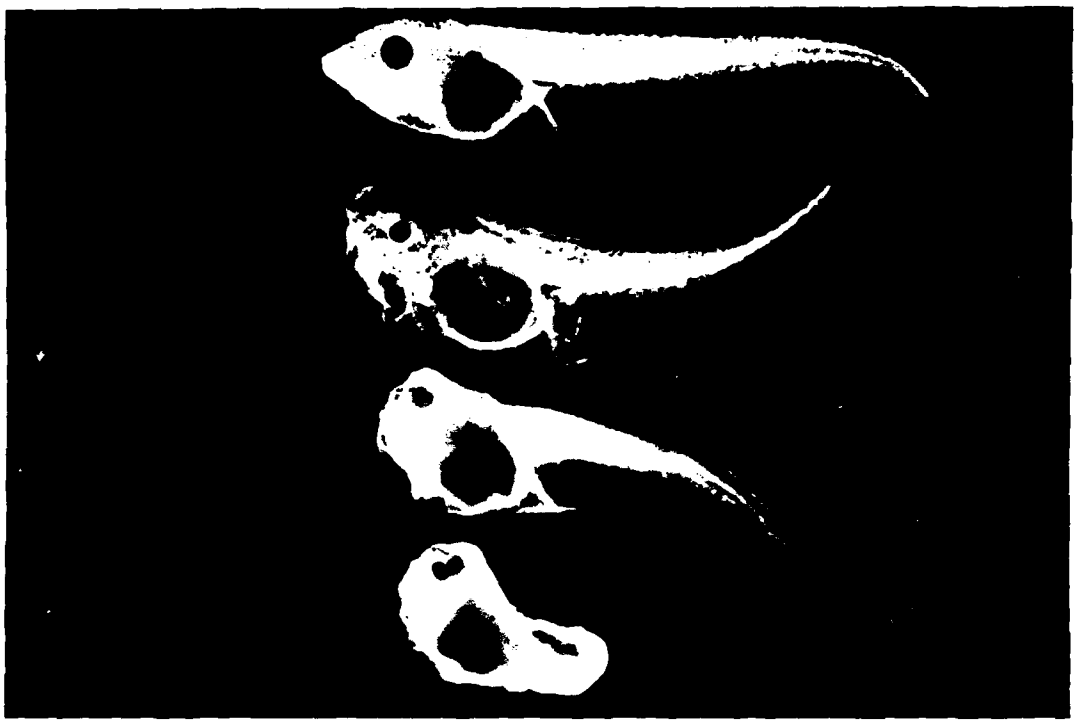

Plate 2B. Effects of Different Concentrations of Aspartame on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, medium concentration, high concentration and very high concentration (near total dead).

5-Azacytidine: 5-Azacytidine is a potent teratogen (34, 36) because it is an inhibitor of nucleic acid synthesis (9). It is not on the Smith list but it causes effects similar to hydroxyurea and 5-Fluorouracil. The latter two compounds are on the Smith list as strong developmental toxicants in animals. We did have some difficulty is performing definitive tests with this compound. Table 1 shows that we were consistent in obtaining 96-hr LC50 values but that we did have problems scoring malformations at certain concentrations and that there was some variability in effect for this endpoint. The TI ranged from a low of 9 to a high of 42 indicative of a strong teratogen (Table 1). Figures 13, 15 and 17 show mortality and malformation dose-response curves for 5-Azacytidine. The mortality curve is clearly separate from the malformation curve and the fit of the data points is excellent. This separation of the curves indicates the teratogenicity of 5-Azacytidine. The effect of 5-Azacytidine on growth is shown in Figures 14, 16, and 18.

The growth inhibition curves shown in Figs. 16 and 18 are nearly identical while Fig 14. is still consistent with the other two graphs. All suggest that 5-Azacytidine is a strong teratogen because there is a significant growth reduction at concentrations that are less than 20% of the 96-hr LC50, a sharp slope in the curve and a greater than 20% decrease in body length at the highest concentrations. All of these are good indicators of a strong developmental toxicant that can inhibit growth.

Plates 3A-5B show the effect of 5-Azacytidine on embryo development. All major organ systems seem to be equally involved and the malformations even at relatively low concentrations (Plate 3B-0.06 mg/ml). There are few eye malformations and pigmentation of the eye appears normal. There may be a slight tendency towards failure of the ventral choroid fissure to fuse. There is a size reduction of the eye but it is correlated to body length so it is not abnormal. There is a consistent upward bend to the tail (Plates 3A, 4B and 5A). The tendency towards severe malformations being caused at low concentrations of toxicant is consistent with the hypothesis that 5-Azacytidine is a strong teratogen.

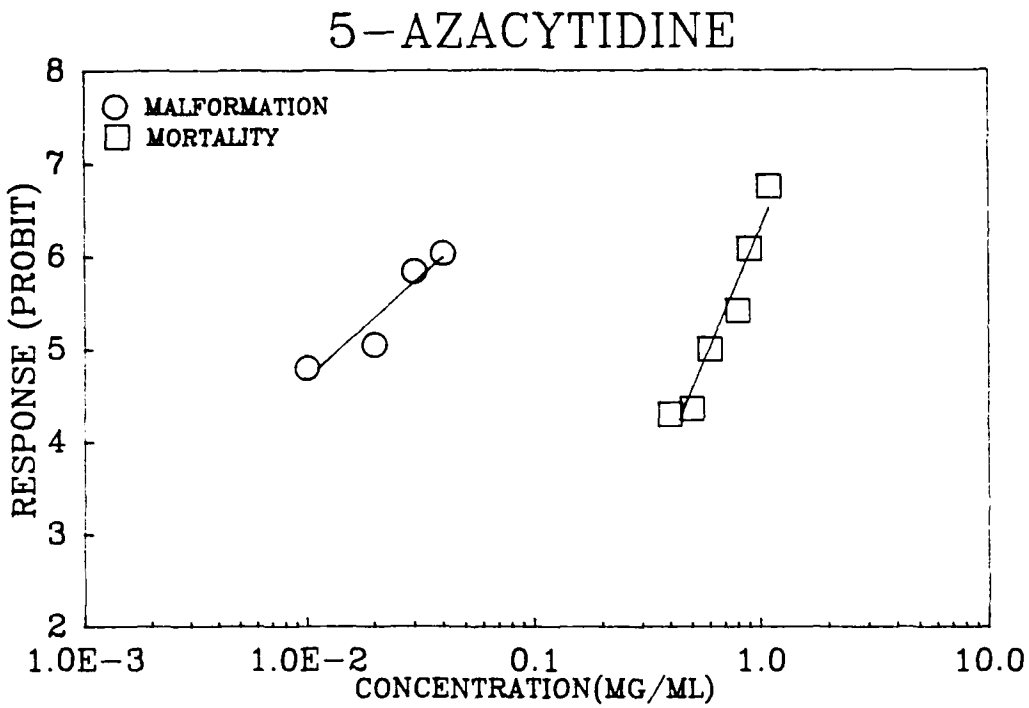

Figure 13. 96-h Mortality and Malformation Dose-Response Curves for 5-Azacytidine Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

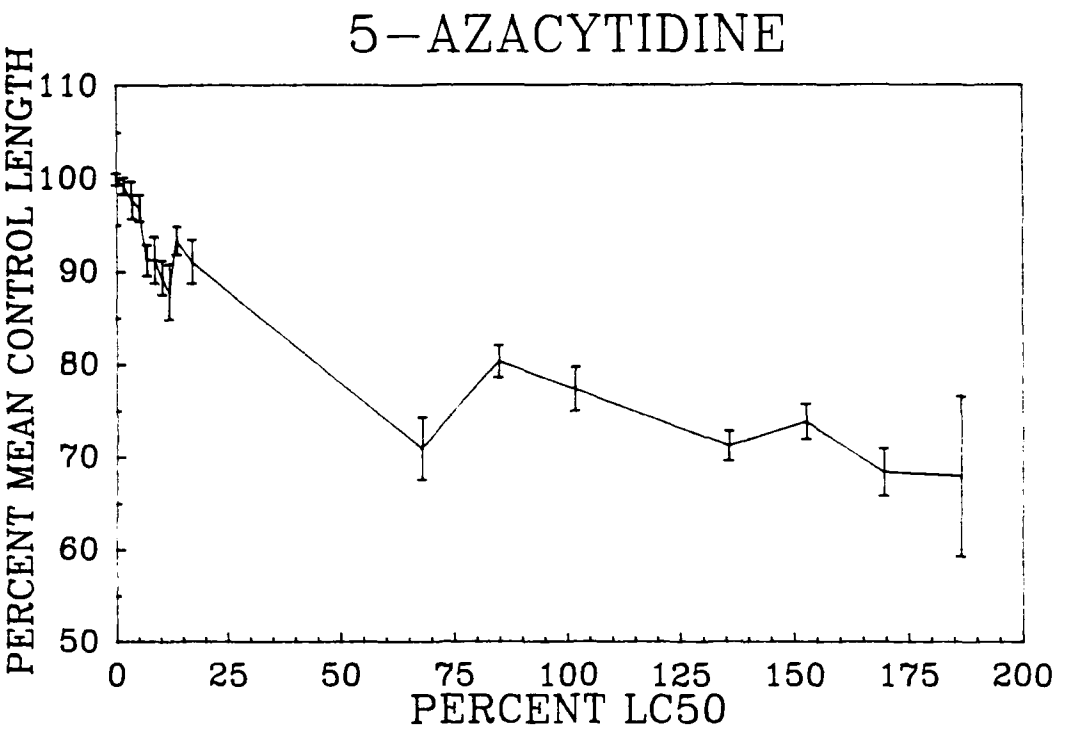

Figure 14. 96-h Growth Dose-Response Curve for 5-Azacytidine Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

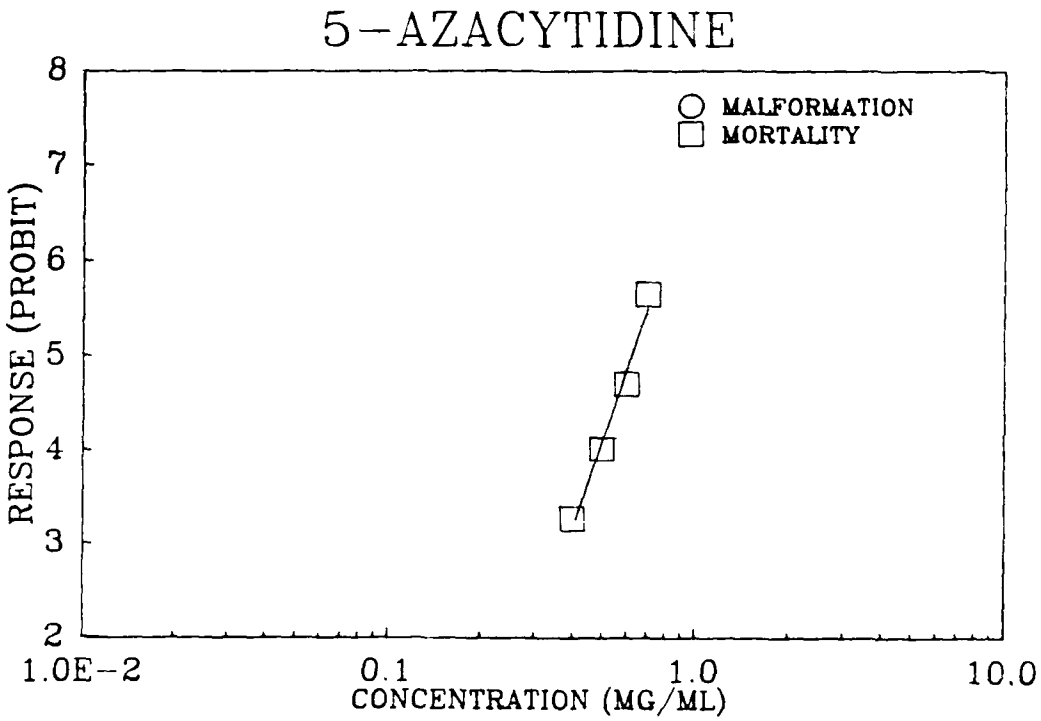

Figure 15. 96-h Mortality and Malformation Dose-Response Curves for 5-Azacytidine Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

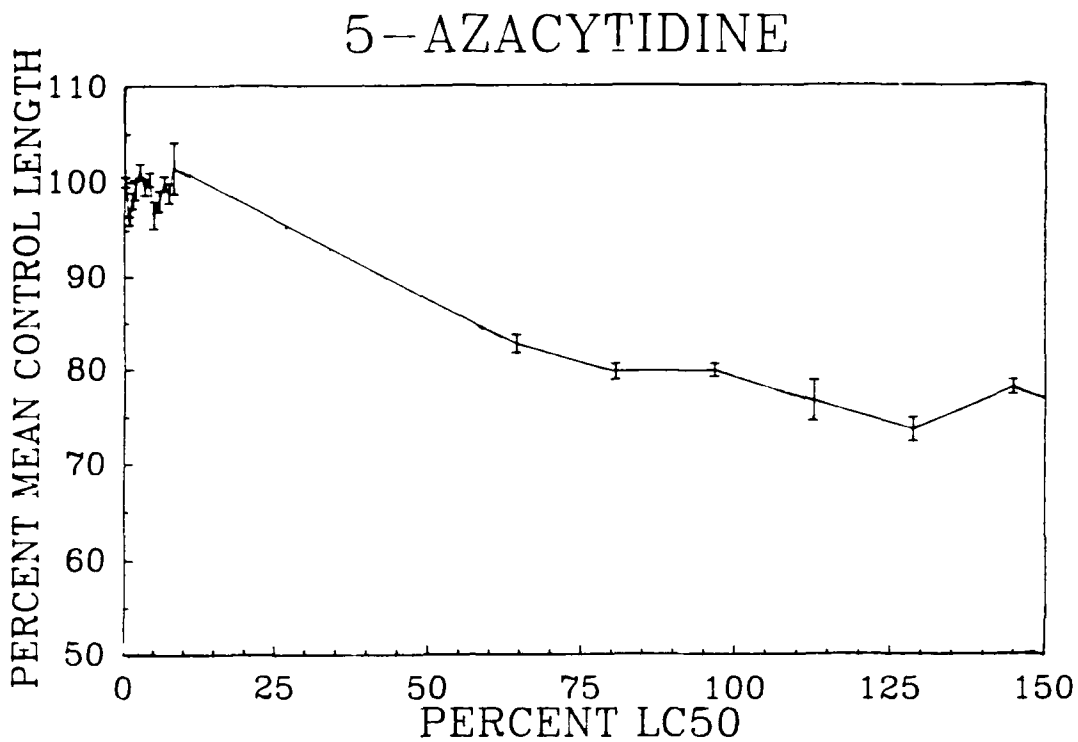

Figure 16. 96-h Growth Dose-Response Curve for 5-Azacytidine Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

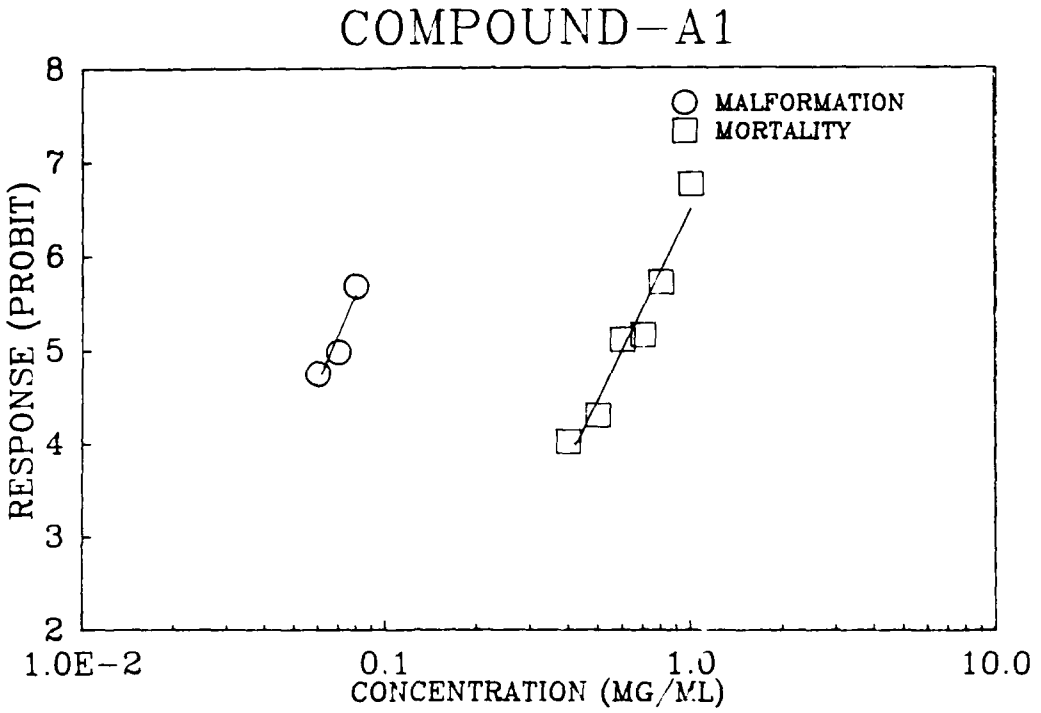

Figure 17. 96-h Mortality and Malformation Dose-Response Curves for 5-Azacytidine Definitive Test #3. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

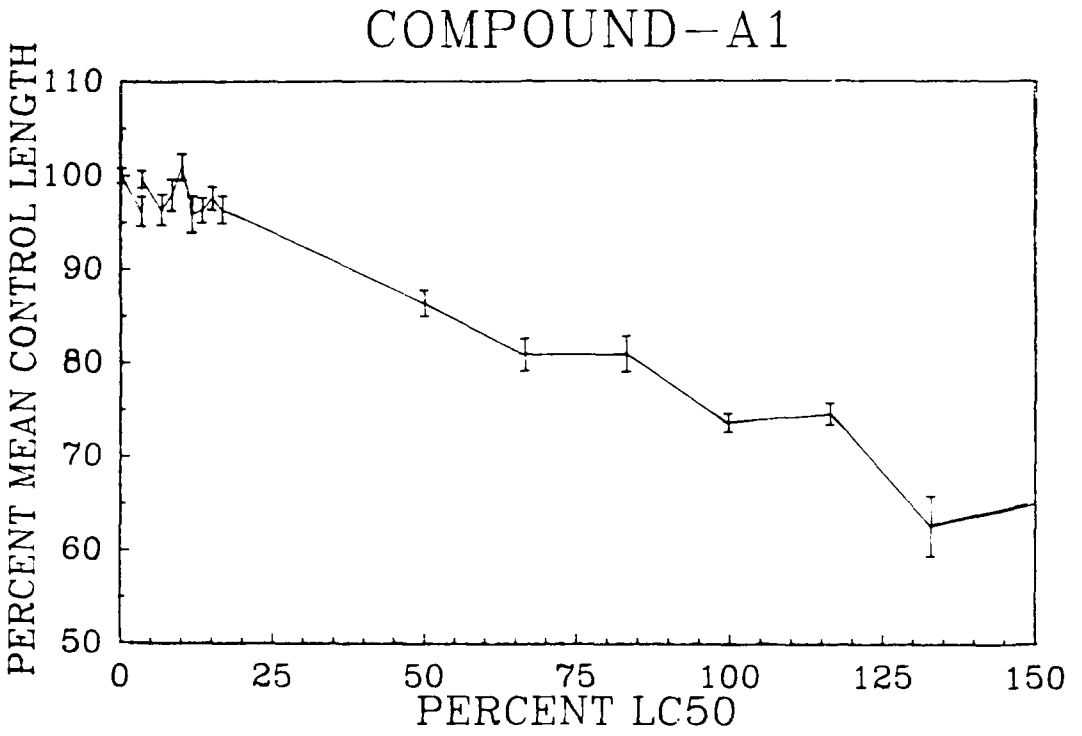

Figure 18. 96-h Growth Dose-Response Curve for 5-Azacytidine Definitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

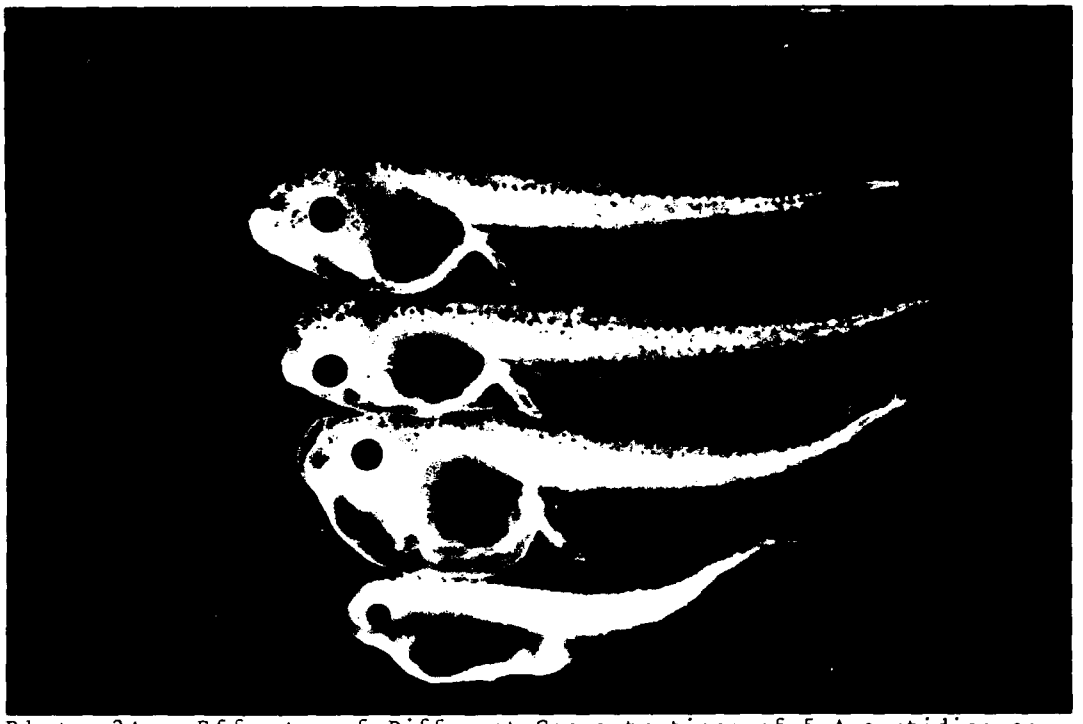

Plate 3A. Effects of Different Concentrations of 5-Azacytidine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 0.06 mg/ml, 0.3 mg/ml, 0.8 mg/ml.

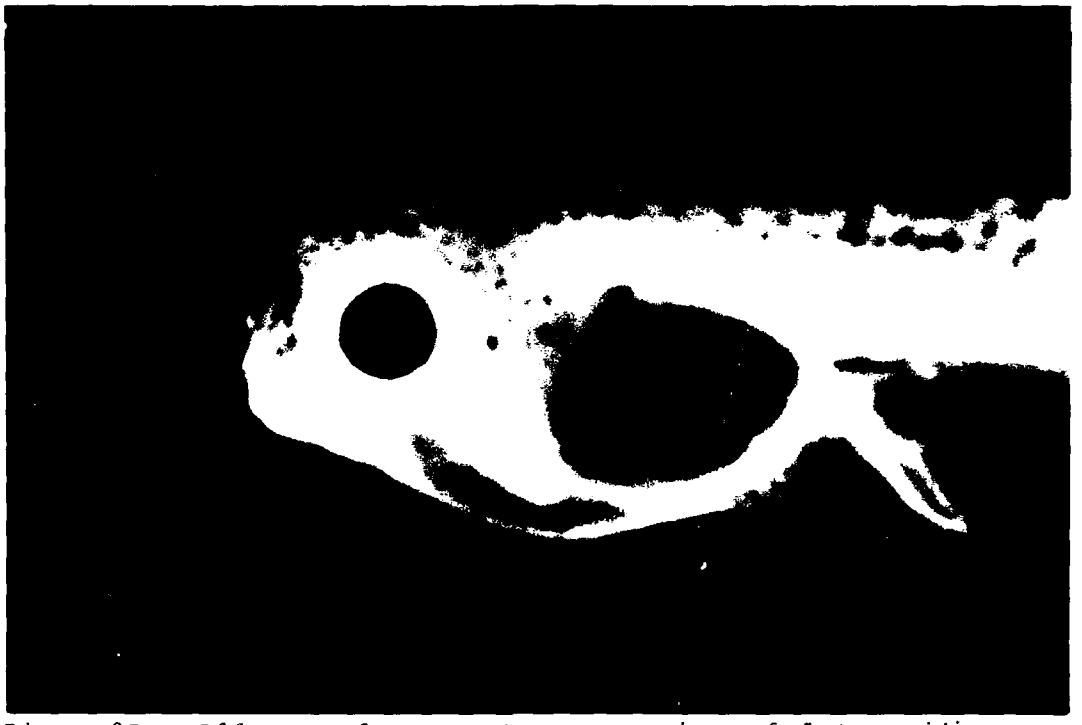

Plate 3B. Effects of a Low Concentration of 5-Azacytidine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show effect on brain and eye region. Embryo exposed to 0.06 mg/ml 5-Azacytidine.

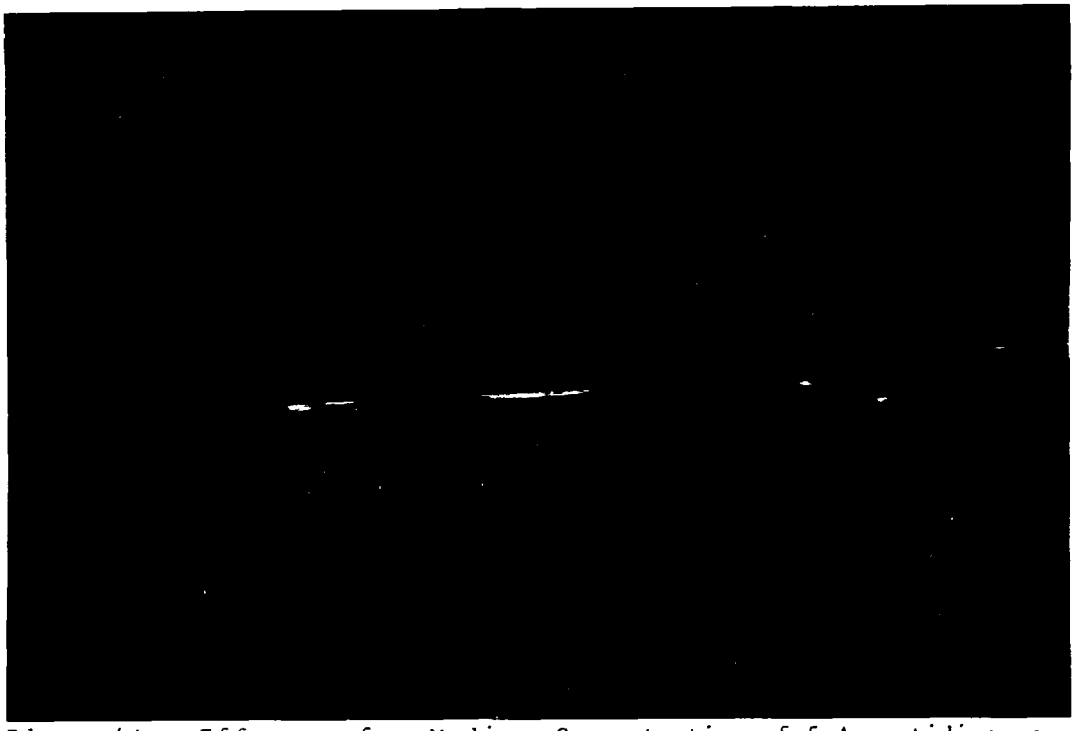

Plate 4A. Effects of a Medium Concentration of 5-Azacytidine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show effect on brain and eye region. Embryo exposed to 0.3 mg/ml 5-Azacytidine.

Plate 4B. Effects of a High Concentration of 5-Azacytidine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show effect on brain and eye region. Embryo exposed to 0.8 mg/ml 5-Azacytidine.

Plate 5A. Effects of Different Concentrations of Methotrexate on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Refer to Plate 4A for control embryo. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: 0.01 mg/ml, 0.05 mg/ml, 0.5 mg/ml.

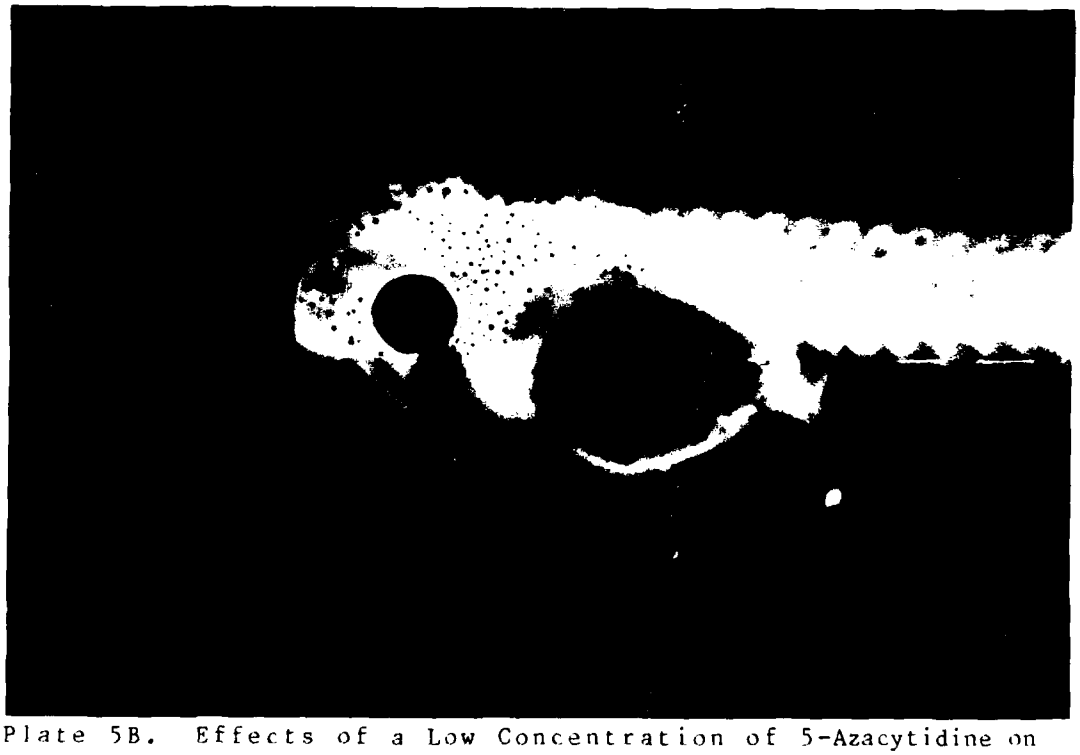

Plate 5B. Effects of a Low Concentration of 5-Azacytidine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show effect on brain and eye region. Embryo exposed to 0.01 mg/ml Methotrexate.

Methotrexate: Methotrexate is a non-variable positive teratogen that acts as a folic acid antagonist and a nucleic acid synthesis inhibitor. It is listed on the Smith list (22) as a positive in both human and animal studies. This assessment is confirmed by all of the citations listed in Shepard (34). Sabourin and Faulk (14) obtained a TI between 1.6-2.5 in FETAX while Dumont obtained a TI of 5.94. We obtained a TI of 43 in the first range test and 23 in the first definitive. We just completed a second definitive test on Methotrexate this week and obtained a TI of 17 with excellent dose-response data. These values are higher than that of Dumont who reported a TI 5.94 for methotrexate. Figures 19 and 21 show the mortality and malformation dose-response curves for Methotrexate. The curve for Fig 19 is a range finder so the points on the curve are few in number and widely separated. The indication is clear from both Figures that there is a wide separation between the two curves. This separation is borne out by the high TI value and suggests that Methotrexate is a strong teratogen.

Growth inhibition by Methotrexate is shown in Figs. 20 and 22. As in the case of 5-Azacytidine, there is a significant growth reduction at concentrations that are less than 20% of the 96-hr LC50, a sharp slope in the curve and a greater than 20% decrease in body length at the highest concentrations. All of these characteristics are good indicators of a strong developmental toxicant that can inhibit growth.

Plate 6A and B shows the effect of both medium and high concentrations of Methotrexate on Xenopus development at 96 hr. As is true for most inhibitors of nucleic acid synthesis, most of the organs of the body are affected. The eyes do not suffer great effects but reduced head size is apparent along with facial malformations. Cardiac malformations are common as is miscoiling of the gut. Plate 6B shows are very abnormal looking embryo exhibiting an upward kinked tail and disrupted myomeres.

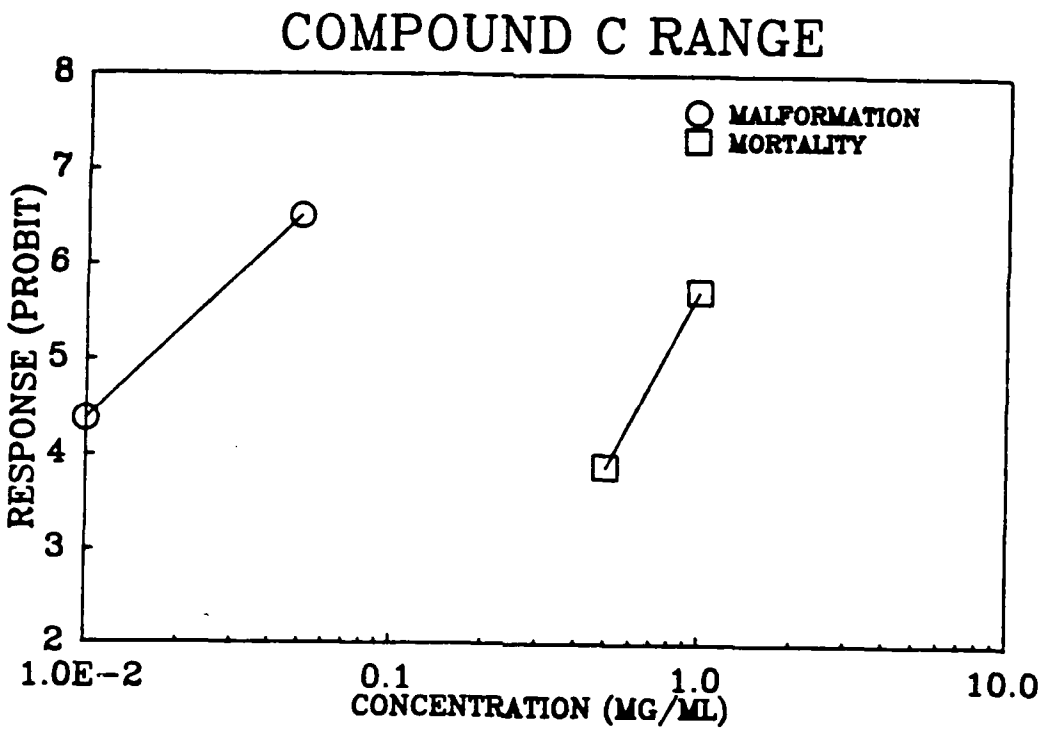

Figure 19. 96-h Mortality and Malformation Dose-Response Curves for Methotrexate Range Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

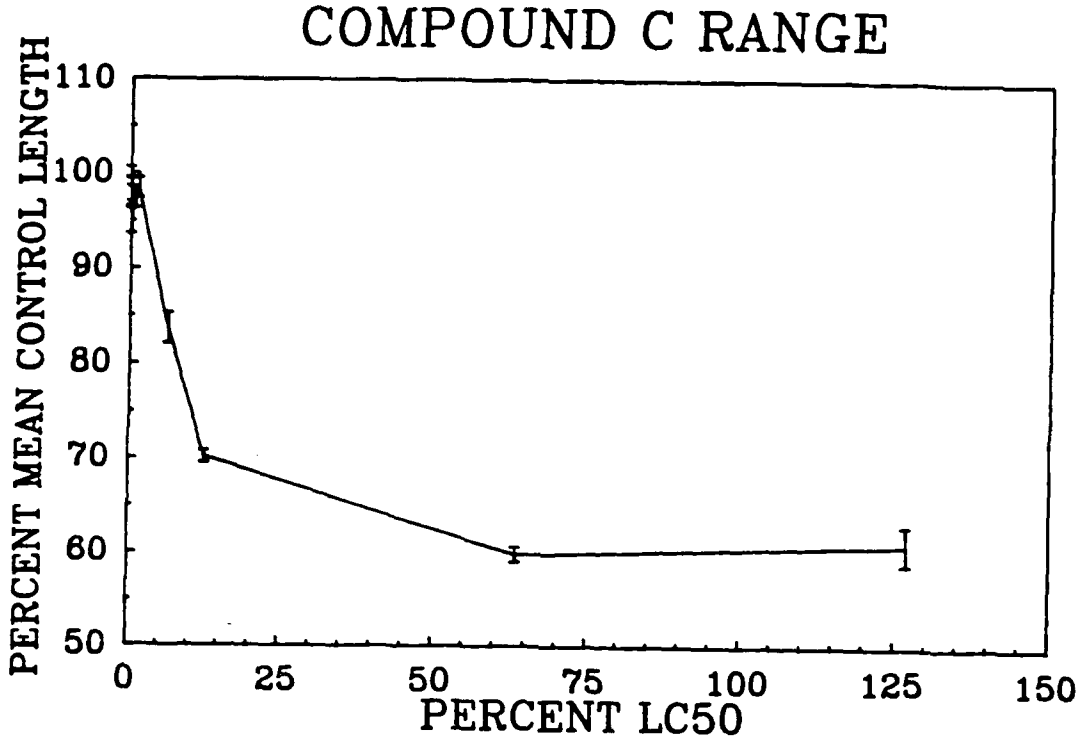

Figure 20. 96-h Growth Dose-Response Curve for Methotrexate Range Test #1. The plot shows the % of control *versus* the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

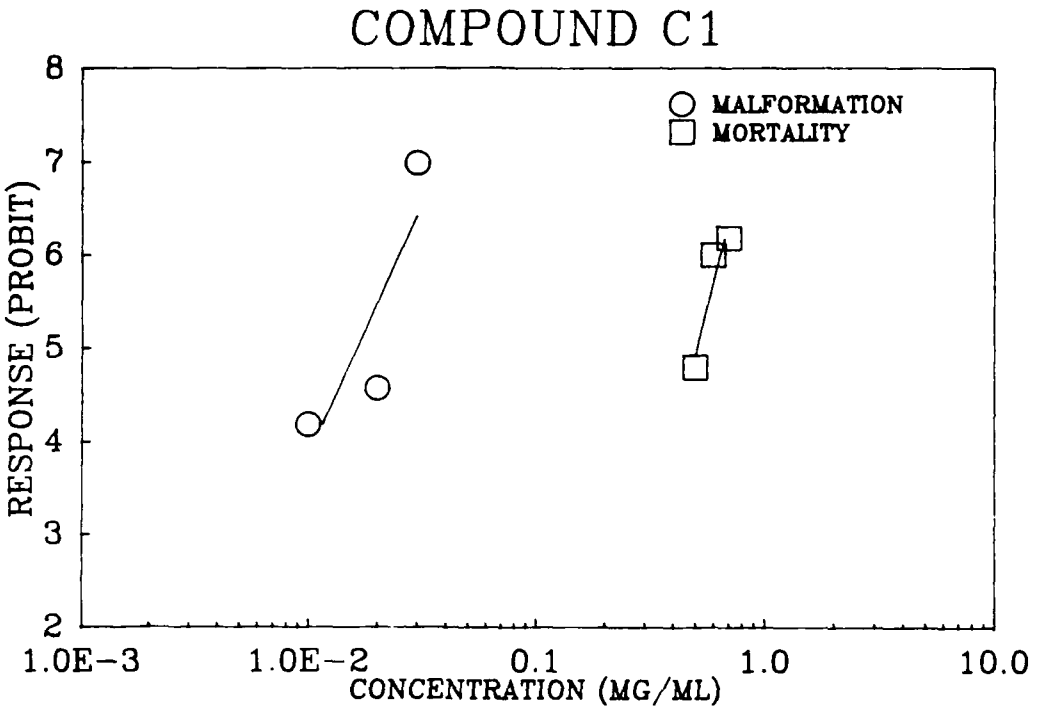

Figure 21. 96-h Mortality and Malformation Dose-Response Curves for Methotrexate Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

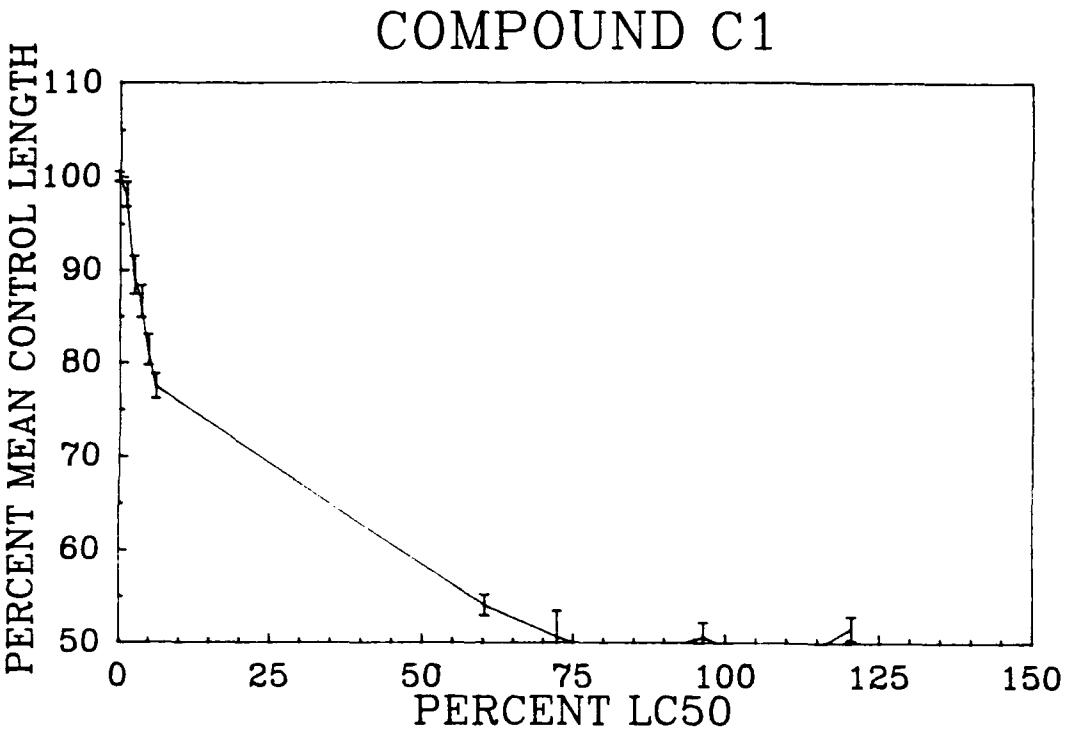

Figure 22. 96-h Growth Dose-Response Curve for Methotrexate, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

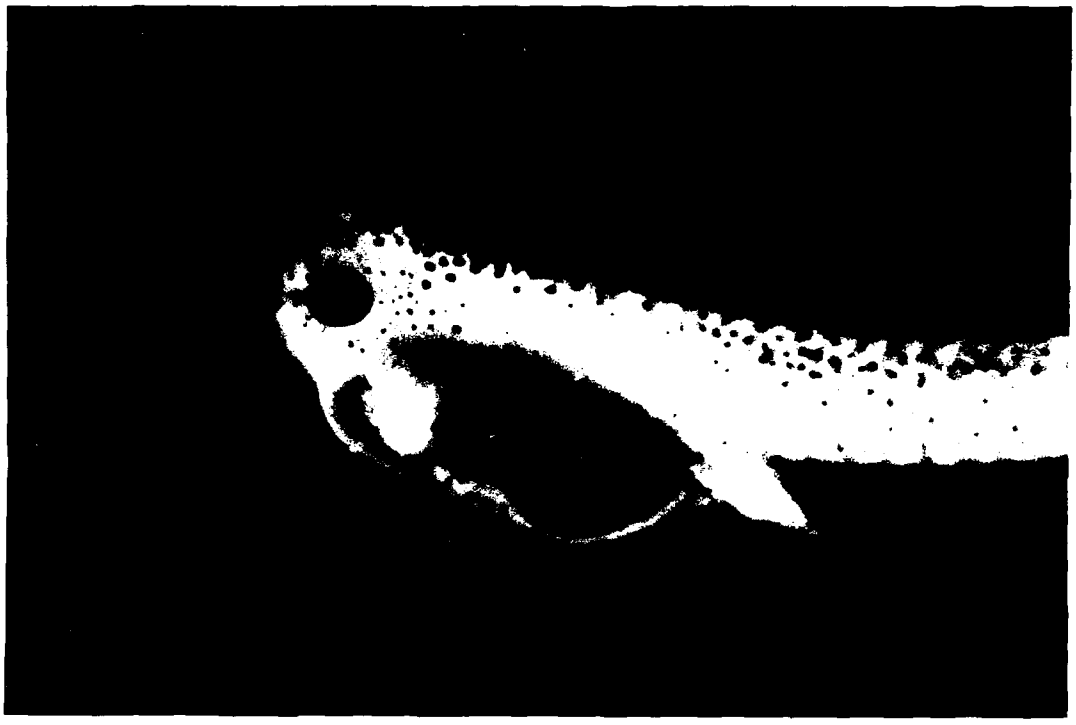

Plate 6A. Effects of a Medium Concentration of Methotrexate on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.05 mg/ml Methotrexate.

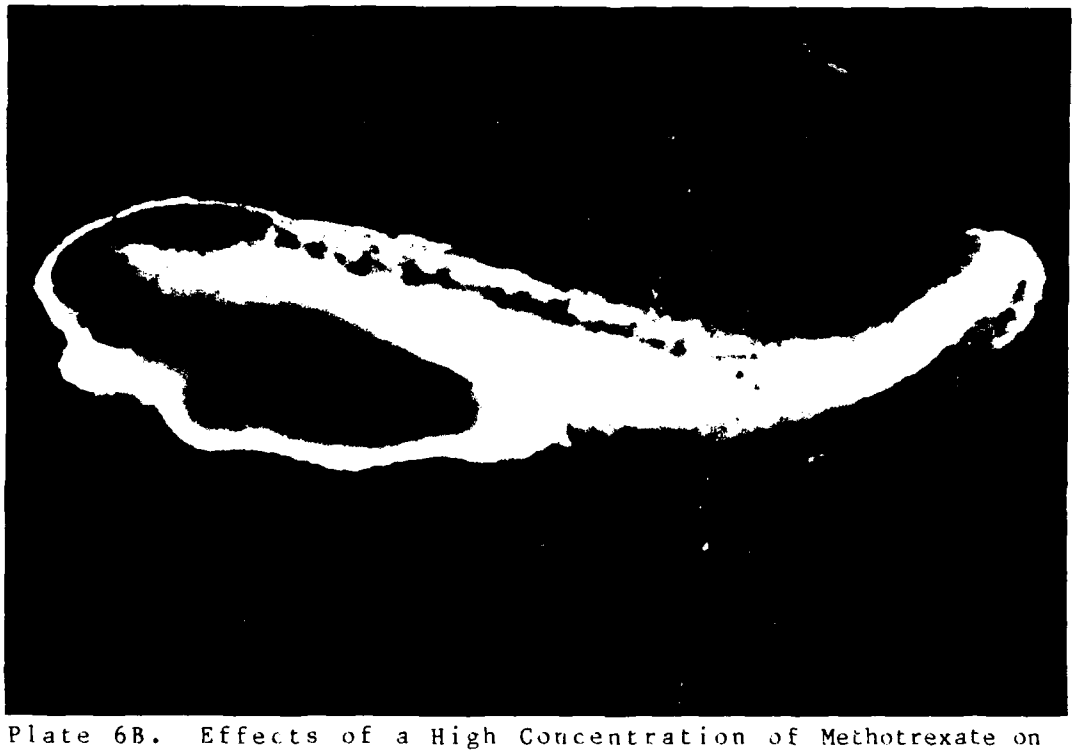

Plate 6B. Effects of a High Concentration of Methotrexate on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.5 mg/ml Methotrexate.

d-Pseudoephedrine: The reports listed in Shepard (34) suggested that d-Pseudoephedrine was a negative. While Epinephrine, a related compound, was a developmental toxicant, Ephedrine, a compound more closely related to d-Pseudoephedrine, did not share this developmental toxicity. Table 1 shows that without metabolic activation (first listing) the mean TI for d-Pseudoephedrine was 1.8. We consider a compound having a TI value over 1.5 to have developmental toxicity and all three TI values listed in Table 1 are above 1.5. Additionally, the 96-hr LC and EC50 values are very close to one another indicating that we were able to precisely repeat these experiments. The three mortality-malformation curves (Figs. 23, 25, and 27) for d-Pseudoephedrine clearly show separation between them. The excellent fit of the data points indicated that the regression lines accurately reflected the dose-response curve. When we obtain results that were not anticipated, we utilize our in vitro metabolic activation system employing rat liver microsomes. The second listing for d-Pseudoephedrine shows the results from a limited series of experiments. Without metabolic activation, we essentially repeated the results of the first three experiments. The number of data points did not allow us to confidently predict the TI. With metabolic activation all of the mortality and malformation was eliminated following d-Pseudoephedrine exposure. We interpret this data to mean that d-Pseudoephedrine would not pose any particular problems in in vivo mammalian experiments.

Figures 24, 26 and 28 show the grow inhibition data for d-Pseudoephedrine. The shape of the curve is consistent for either nonteratogen or a weak teratogen. Over the first 20% of the 96-hr LC50 the growth inhibition is much less than 20%. The slope is not too steep compared to Figure 22 for Methotrexate. The overall maximum reduction in growth for d-Pseudoephedrine is about 30-35%. This is more typical of a weak teratogen than a nonteratogen. All three growth inhibition curves are nearly superimposable indicating good precision in the data.

Plates 7 and 8 show the effects of different d-Pseudoephedrine concentrations on Xenopus development after 96 hr of exposure. Plate 7A shows typical embryos from three separate concentrations of d-Pseudoephedrine and a control embryo (top). The embryo from the 0.25 mg/ml concentration shows the effects seen at about the 96 hr EC50 (malformation) (Plate 7b). This embryo exhibits moderate malformations particularly in the head and face regions and this was typical of most of the malformed embryos. Other typical malformations in the concentration range were loose gut coiling and cardiac edema. Many embryos showed blisters as well. In higher concentrations these same abnormalities still dominated except that they were more severe. Plates 8A and 8B show the more severe malformations that occur at higher concentrations. Note that 8A shows an embryo that exhibits failure of the choroid fissure of the eye to fuse at the ventral aspect. Ocular and cardiac edema are apparent. Plate 8B shows a ventral view of an embryo near the 96-hr LC50. The gut is distended and not well coiled. Ocular edema is obvious and there are blisters near the anus.

In summary, d-Pseudoephedrine is a weak to moderate teratogen unless there is metabolic breakdown present. It then rapidly loses much of its embryotoxicity and teratogenicity. Without the metabolic activation system d-Pseudoephedrine would have been scored as a false positive in FETAX.

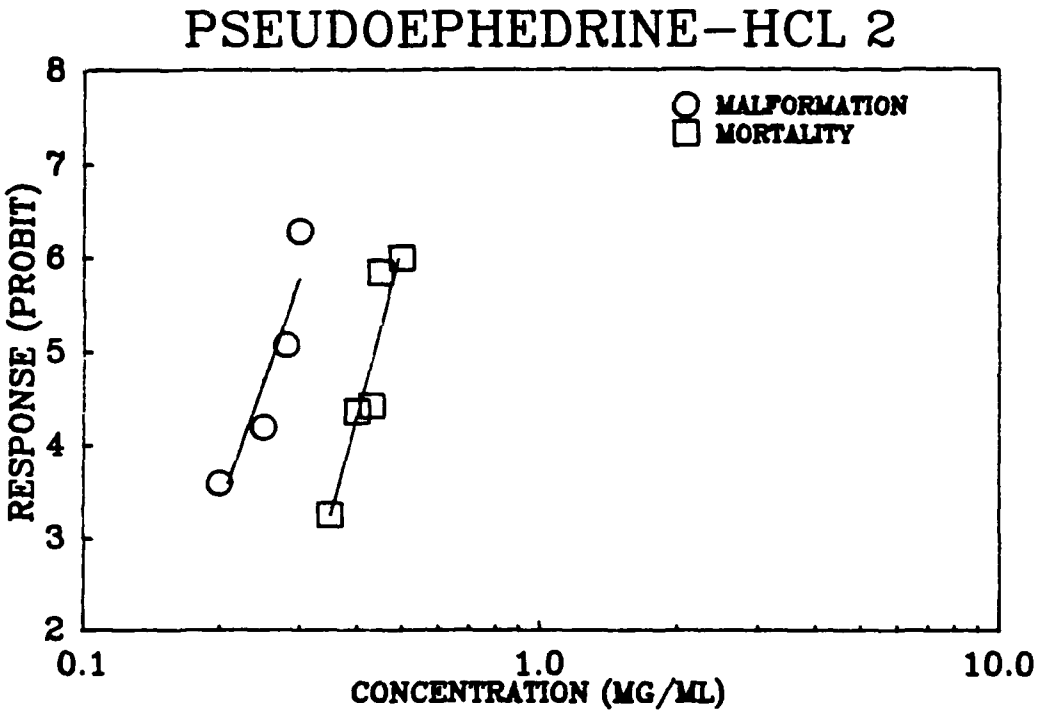

Figure 23. 96-h Mortality and Malformation Dose-Response Curves for Pseudoephedrine Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

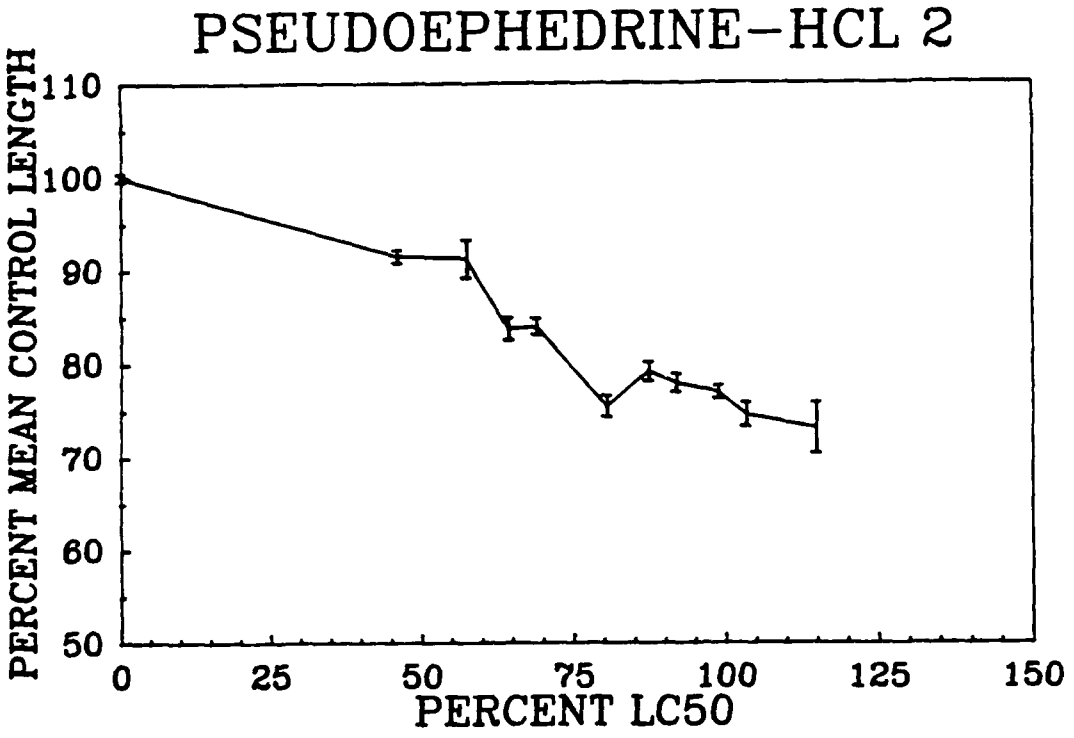

Figure 24. 96-h Growth Dose-Response Curve for Psuedoephedrine Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

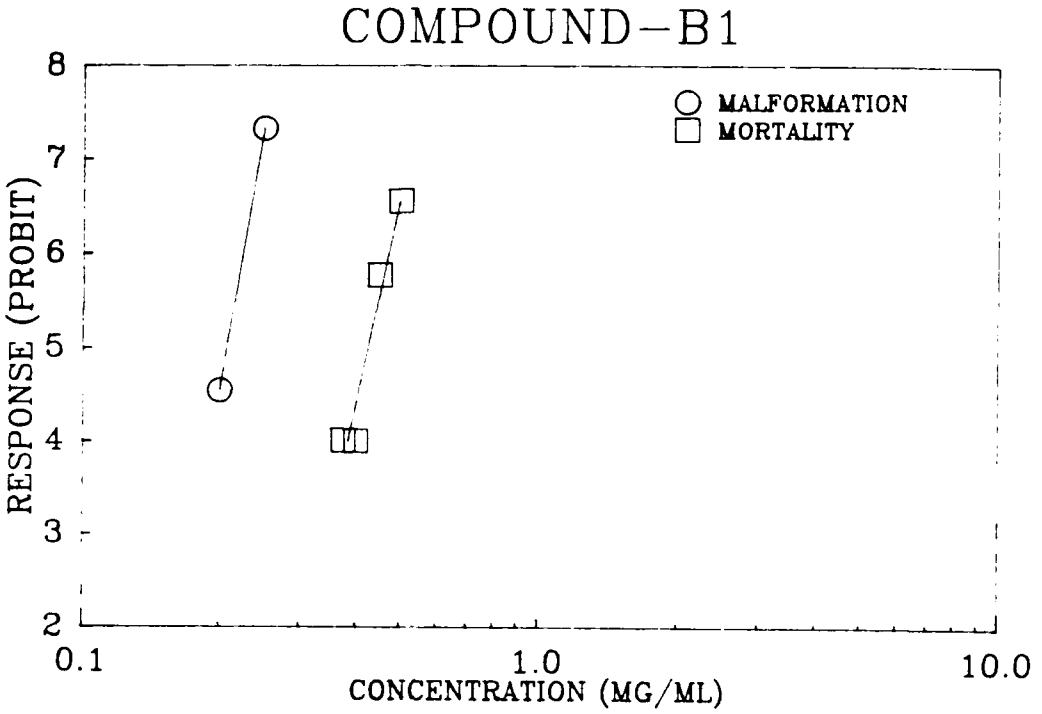

Figure 25. 96-h Mortality and Malformation Dose-Response Curves for Psuedoephedrine Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

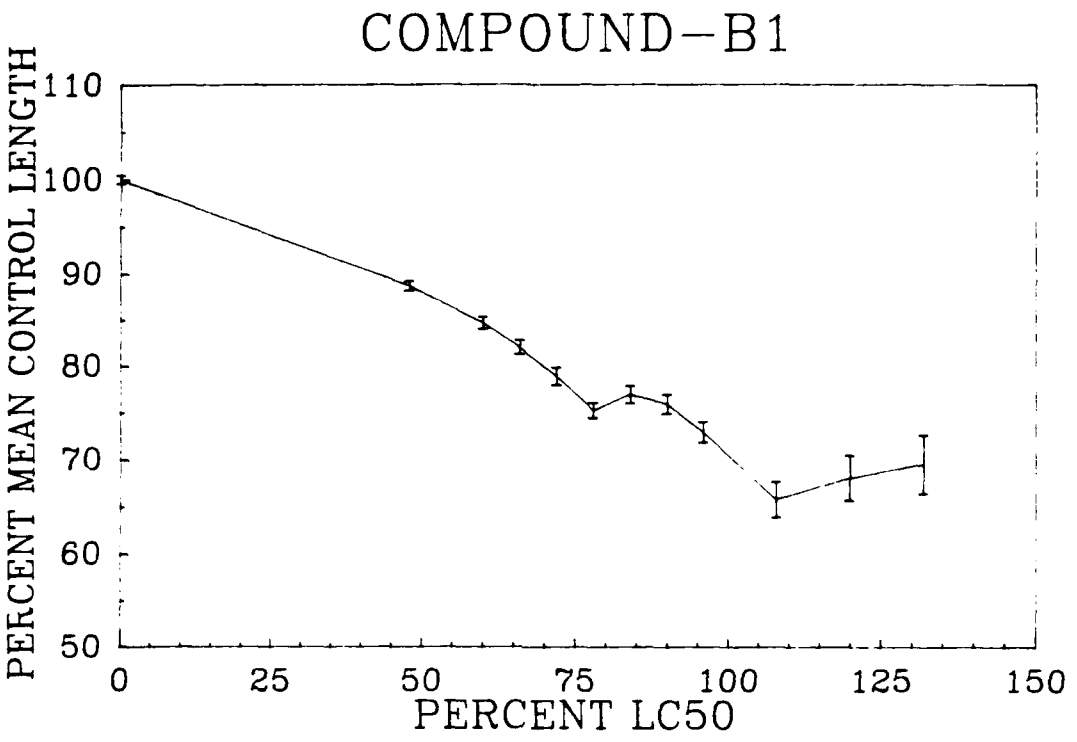

Figure 26. 96-h Growth Dose-Response Curve for Psuedoephedrine Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

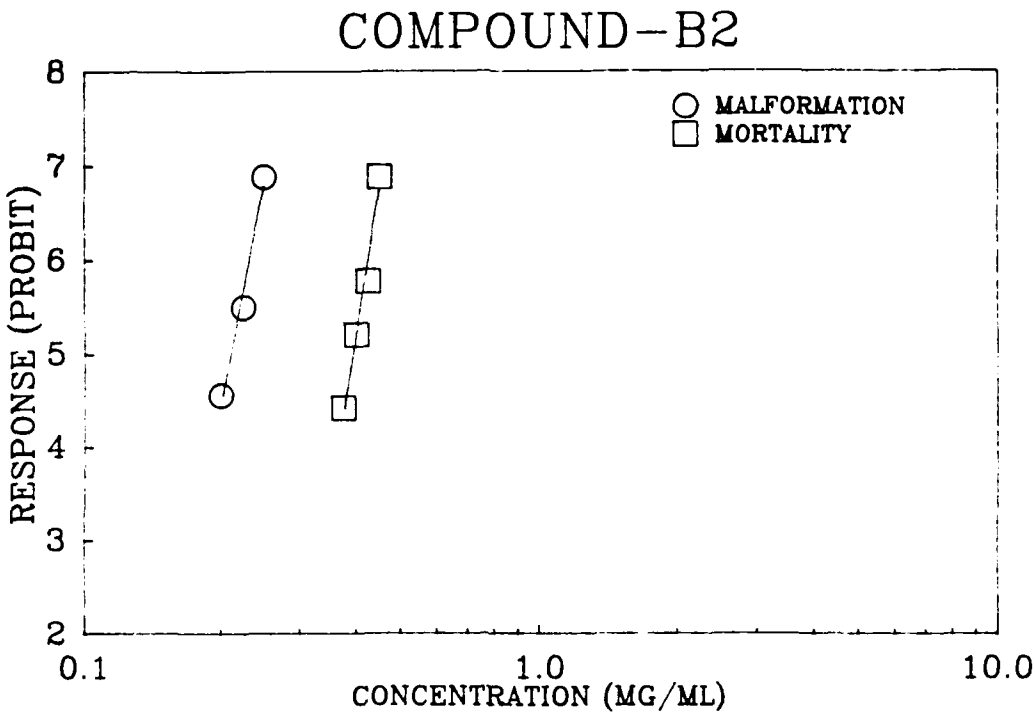

Figure 27. 96-h Mortality and Malformation Dose-Response Curves for Psuedoephedrine Definitive Test #3. . The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

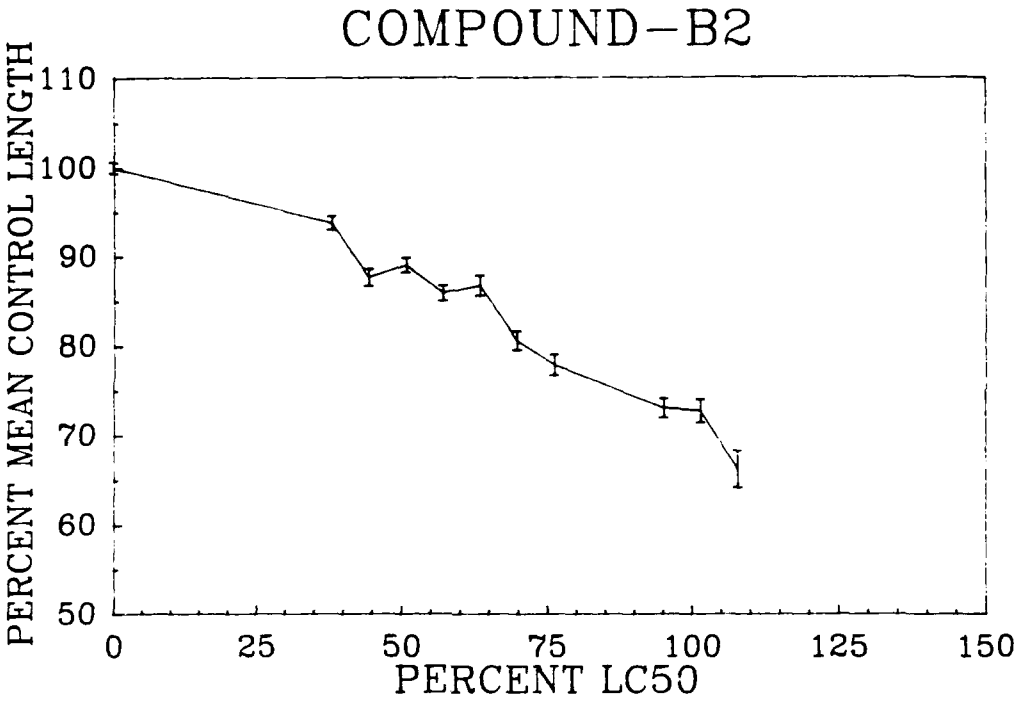

Figure 28. 96-h Growth Dose-Response Curve for Psuedoephedrine Definitive Test #3. . The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

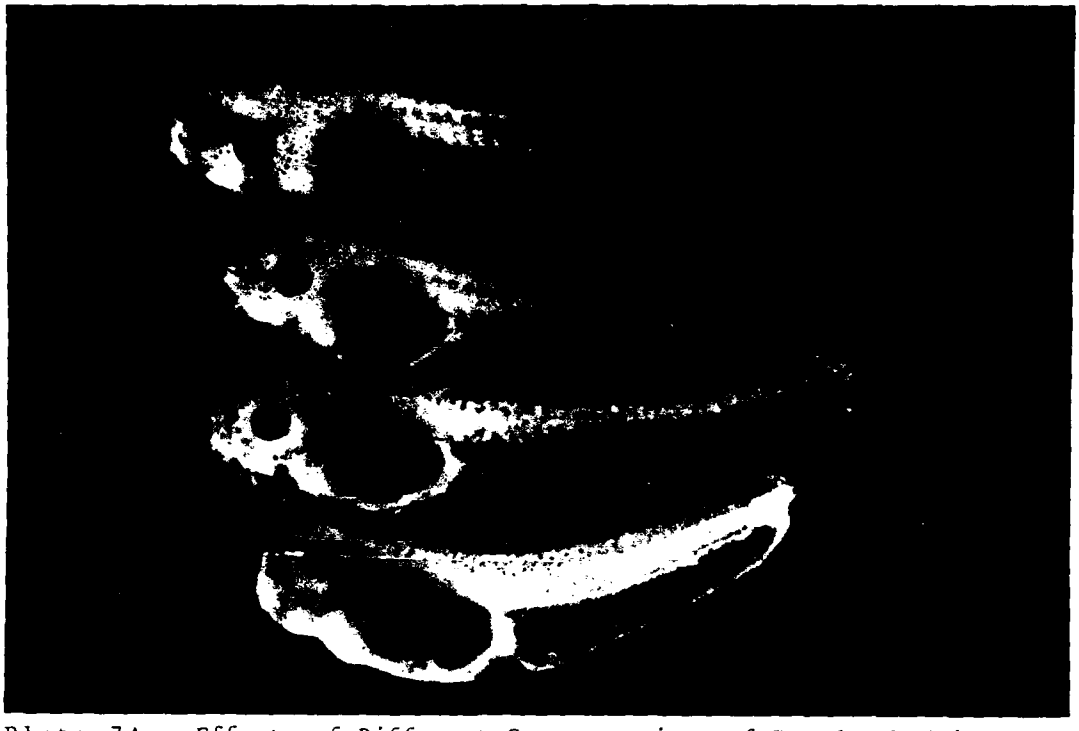

Plate 7A. Effects of Different Concentrations of Pseudoephedrine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 0.25 mg/ml, 0.3 mg/ml, 0.5 mg/ml.

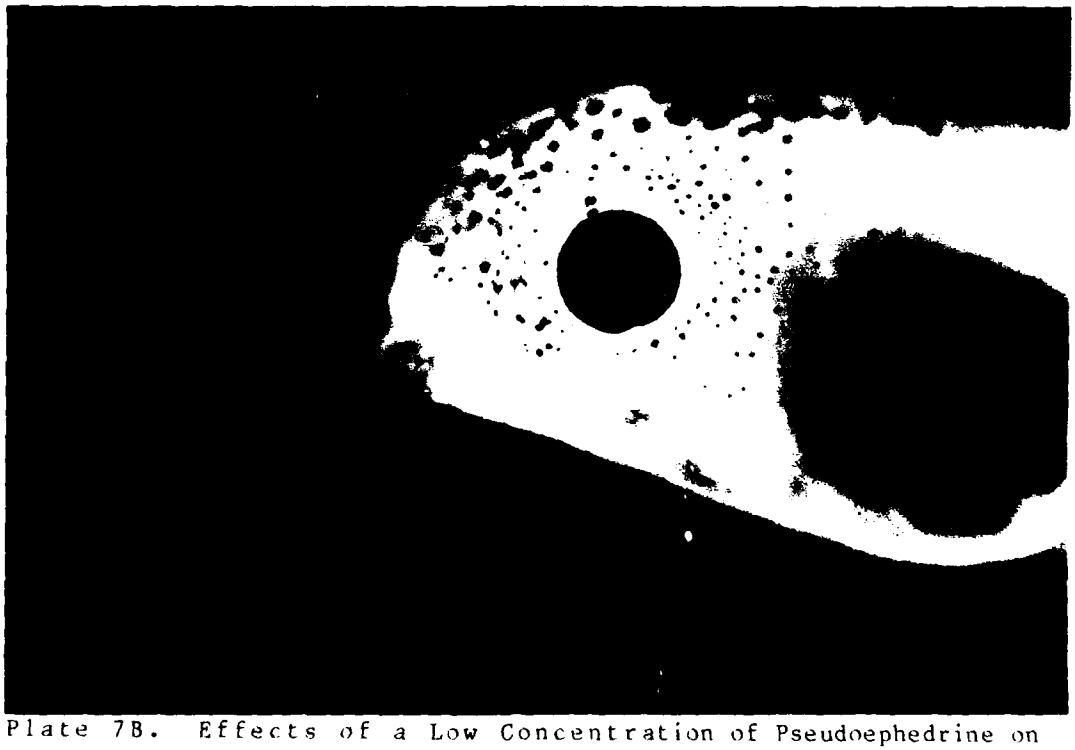

Plate 7B. Effects of a Low Concentration of Pseudoephedrine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.25 mg/ml Pseudoephedrine.

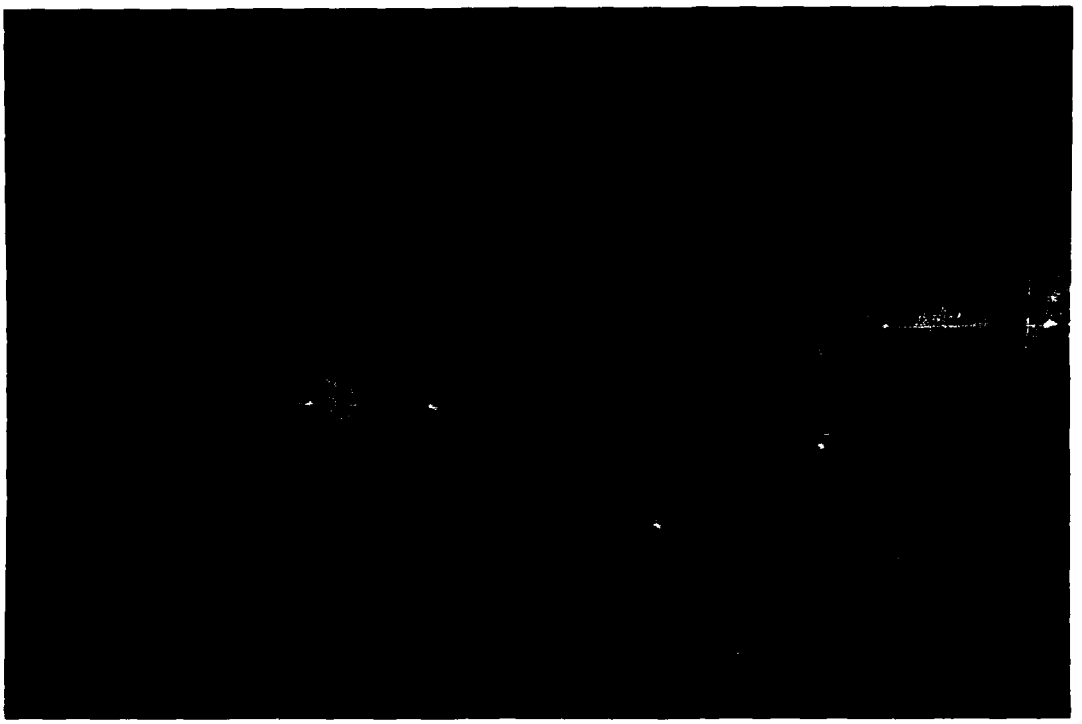

Plate 8A. Effects of a Medium Concentration of Pseudoephedrine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.3 mg/ml Pseudoephedrine.

Plate 8B. Effects of a High Concentration of Pseudoephedrine on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged ventral view presented to show the effect on brain and eye region. Embryo exposed to 0.5 mg/ml Pseudoephedrine.

2. Solvent Interaction Study

Many insoluble compounds cannot presently be tested in FETAX unless it is first dissolved in a carrier solvent. While it is possible to determine the NOEC for solvents such as Triethylene Glycol, Acetone and Dimethyl Sulfoxide, there is still a possible positive or negative interaction with the test compound that could alter test results. There is ample evidence of solvent interaction in the literature. Nelson et al. (37) showed that ethanol reduced the number of neurochemical effects of 2-ethoxyethanol in rats. Demey et al. (38) found that propylene glycol when used in a IV nitroglycerin solution may cause hyperosmolality, hemolysis and lactic acidosis. Gichner and Veleminsky (39) showed that 4-12% acetone, 4-16% ethanol and 8-32% dimethylformamide potentiate the mutagenic activity of N-methyl-N'-nitro- N-nitrosoguanidine in Arabidopsis thaliana seeds. These findings make the present study imperative.

In order to conduct the solvent interaction study it was first necessary to perform dose-response curves for each of the solvents to be used as carriers and to obtain similar data for the compounds to "interact" with the solvents. From these curves the NOEC and 96-hr LC25 and EC50 (malformation) is estimated. It should be remembered that support for the latter dose-response curves is from an Oklahoma Center for the Advancement of Science and Technology grant so detailed data will not be presented here with the exception of trans-retinoic acid in order to conserve space. On the annual report, all of the data will be presented so that conclusions may be drawn.

Triethylene glycol: Triethylene glycol (TG) is very similar to other glycol derivatives but is not as toxic as them. Weyland (40) used it as a vehicle for Benzo(a)pyrene. However, Weyland does not mention any interaction with TG and Benzo(a)pyrene. TG is used as a drying agent for natural gas, in the manufacture of vinyl plasticizers and as a solvent. TG is not as toxic a solvent as DMSO or acetone in FETAX (Table 2). The mean TI of 1.12 indicates that TG is not teratogenic. The mean 96-hr LC50 is 2.45 v/v% making it much less toxic than either Acetone or DMSO (Table 2). The same is true for the 96-hr EC50(malformation) which has a mean of 2.15 v/v%. Figures 29, 31 and 33 show the 96-hr mortality and malformation dose-response curves for TG. The lines are consistently superimposed on one another (or nearly so) indicating a nonteratogen. Replicability is excellent and there are many data points in the partial effects zone of 16-84% which suggests that the data is reliable. Growth inhibition curves (Figures 30, 32, and 34) are repeatable and indicate that TG is a nonteratogen because the are no effects seen until concentrations are reached that are 50% of the 96-hr LC50 or greater. It is interesting that a 40% growth inhibition can be reached in surviving embryos. Plates 9 and 10 show that abnormalities are minor even at concentrations of 2% (Plate 9A and 9B). This called a low concentration in the plate legend but this refers to the other embryos exposed to higher concentrations presented on the Plate. Concentrations of 1 to 1.5% TG would be used as a carrier in FETAX as there are no observable malformations at these concentrations and growth is not affected. At higher concentrations, as shown in Plates 9 and 10, TG causes facial abnormalities and some shifting in the position of the eye. Gut and heart coiling are impaired at higher concentrations and little effect on the tail is seen. While TG proved to be the least toxic and teratogenic of the proposed carrier solvents it is not the best solvent and will not carry all test compounds into solution.

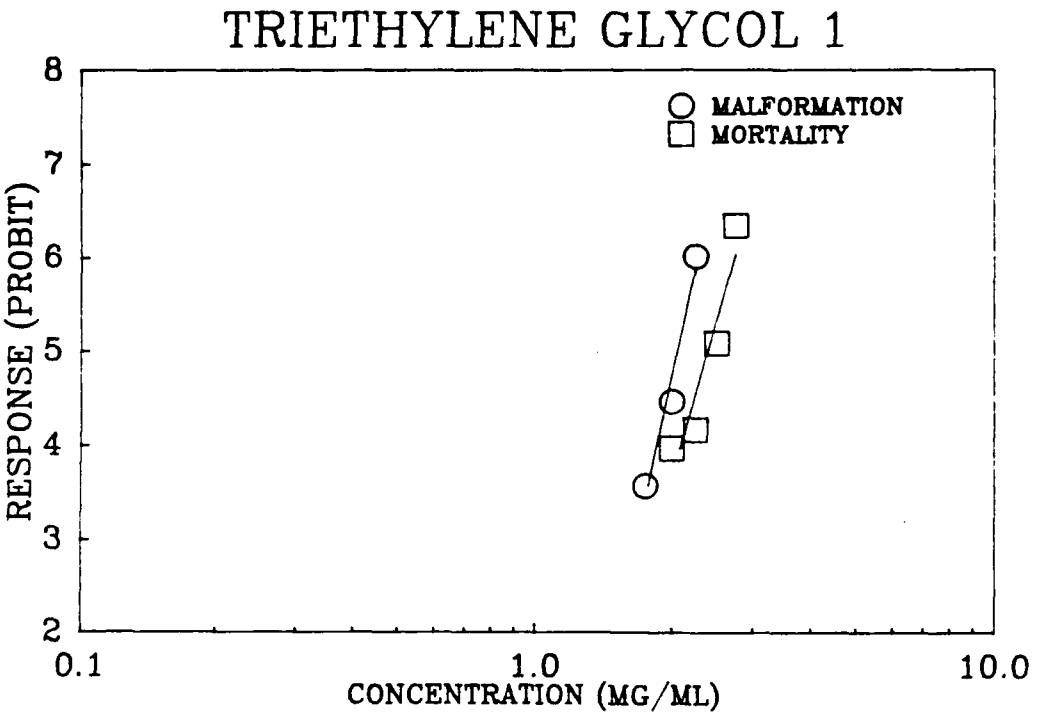

Figure 29. 96-h Mortality and Malformation Dose-Response Curves for Triethylene glycol Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

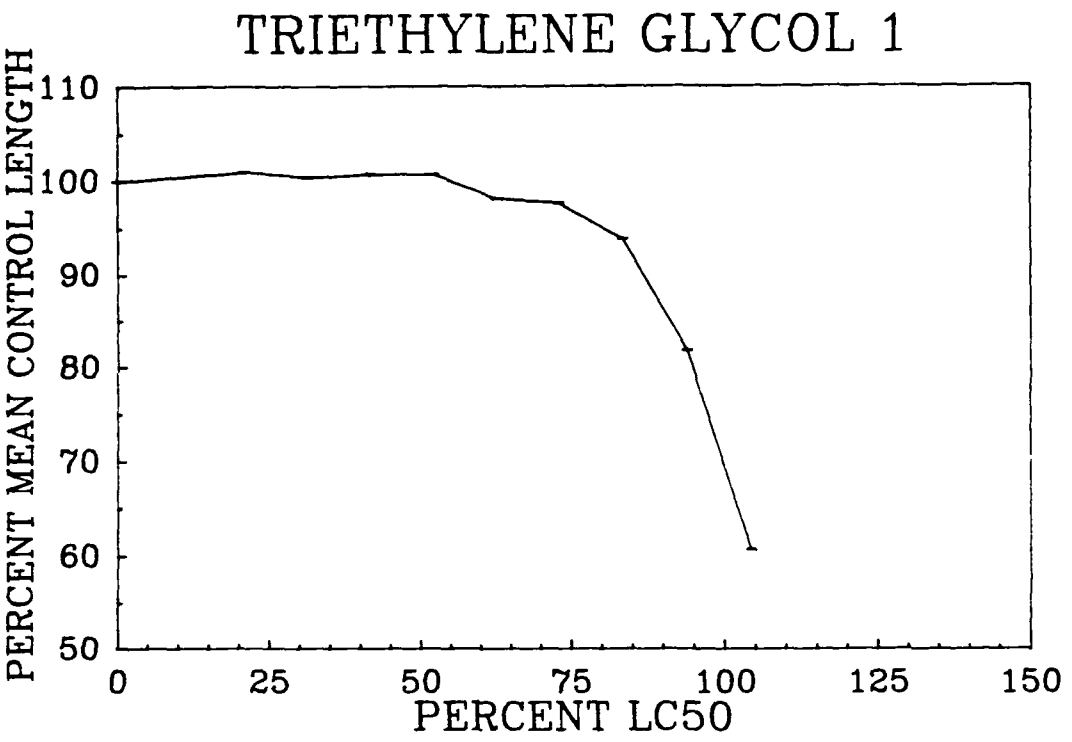

Figure 30. 96-h Growth Dose-Response Curve for Triethylene glycol Definitive Test #1. The plot shows the % of control versus the % of the 96-h *LC50*. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

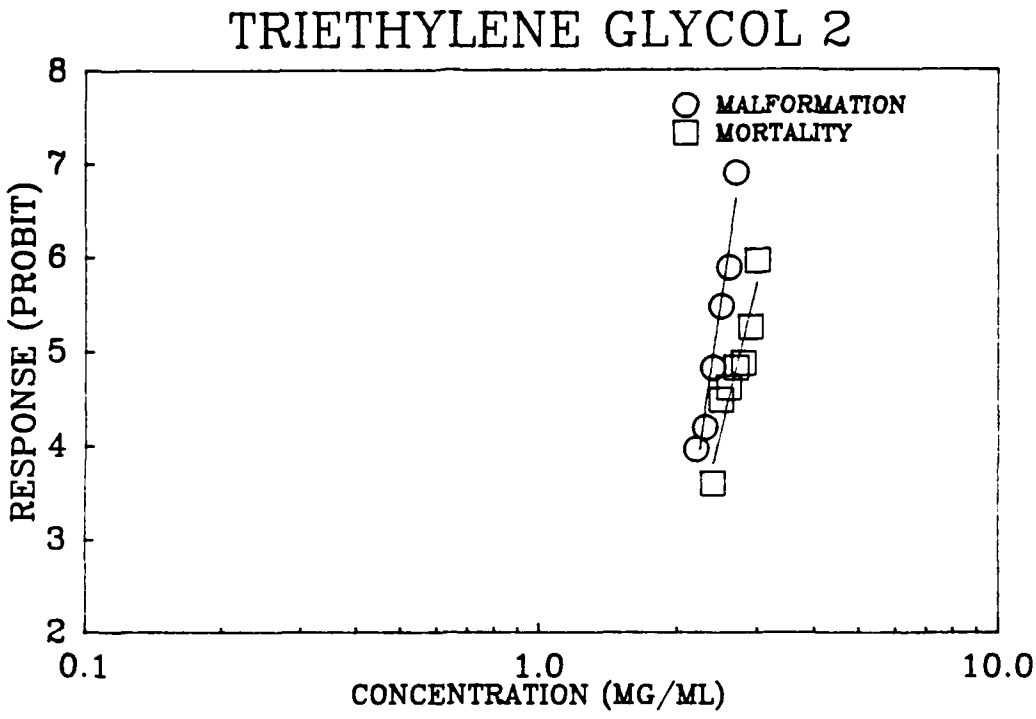

Figure 31. 96-h Mortality and Malformation Dose-Response Curves for Triethylene glycol Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

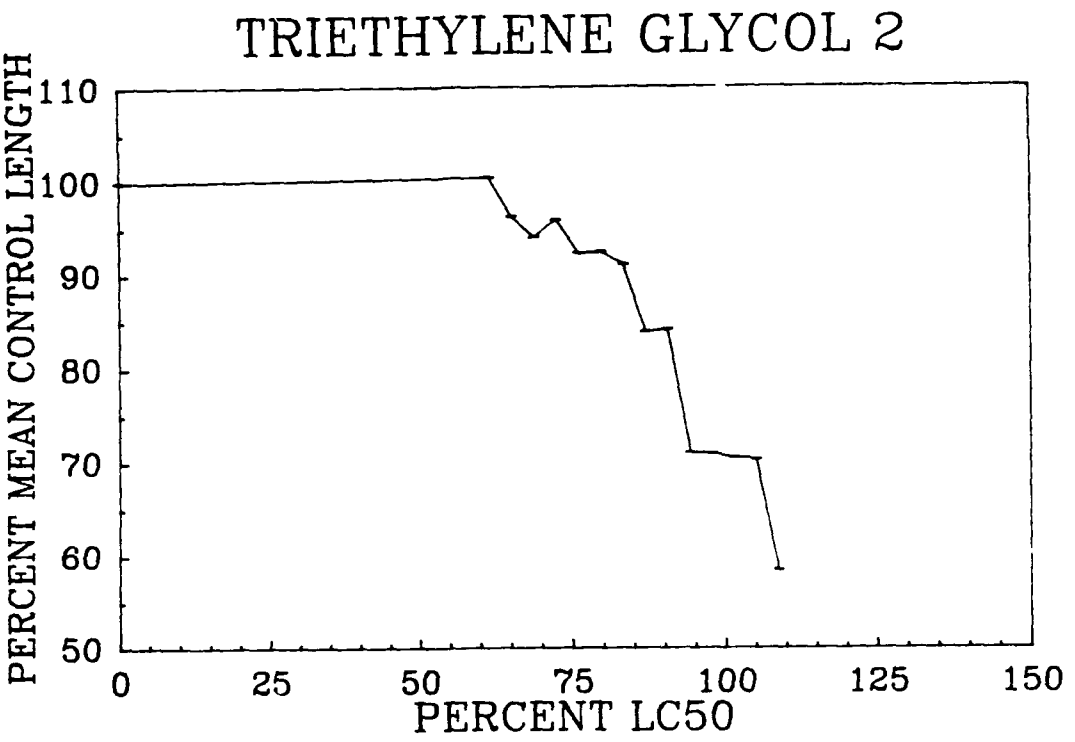

Figure 32. 96-h Growth Dose-Response Curve for Triethylene glycol Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

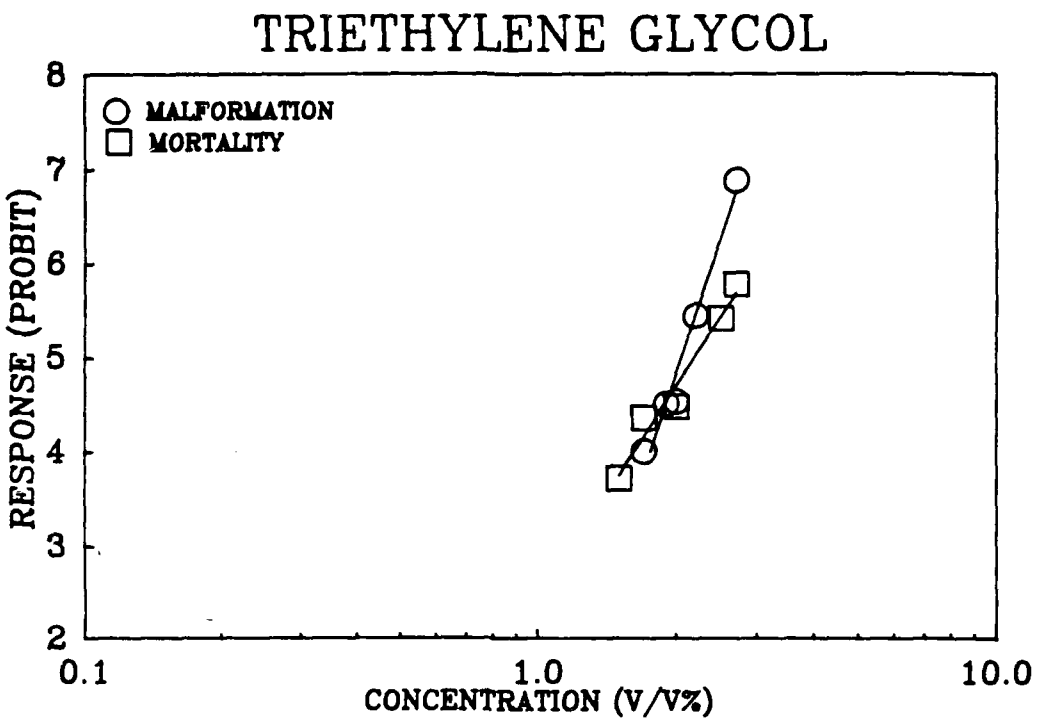

Figure 33. 96-h Mortality and Malformation Dose-Response Curves for Triethylene glycol Definitive Test #3. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

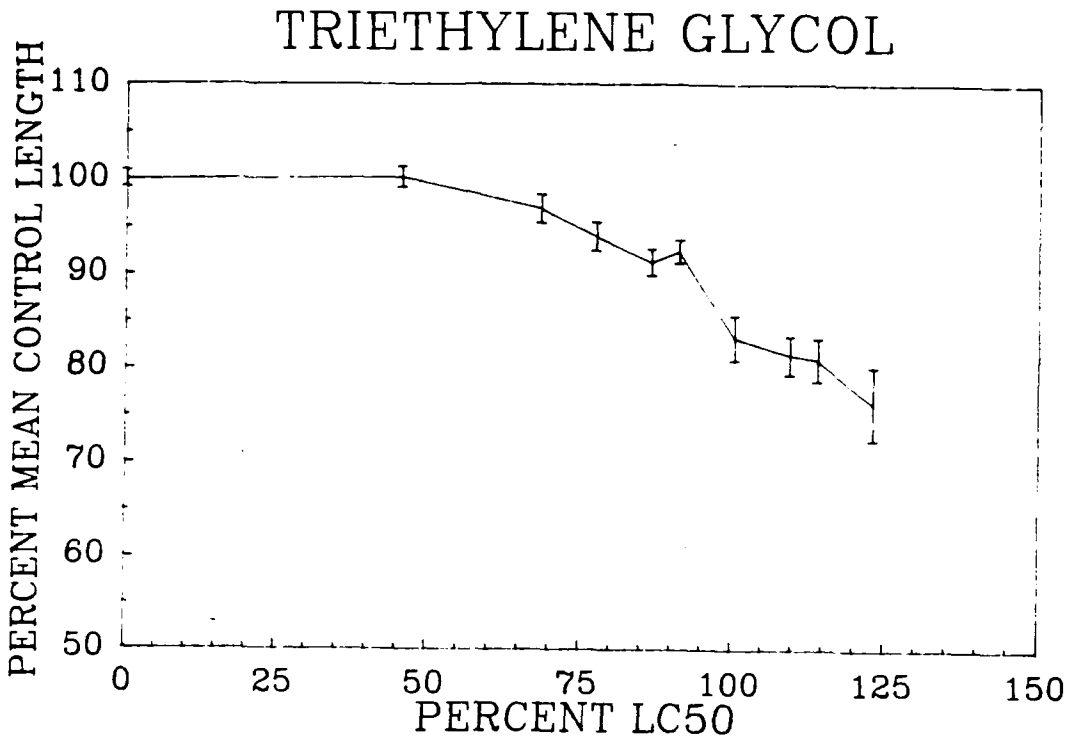

Figure 34. 96-h Growth Dose-Response Curve for Triethylene glycol Definitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

Plate 9A. Effects of Different Concentrations of Triethylene Glycol on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 2% v/v, 2.4% v/v, 2.7% v/v.

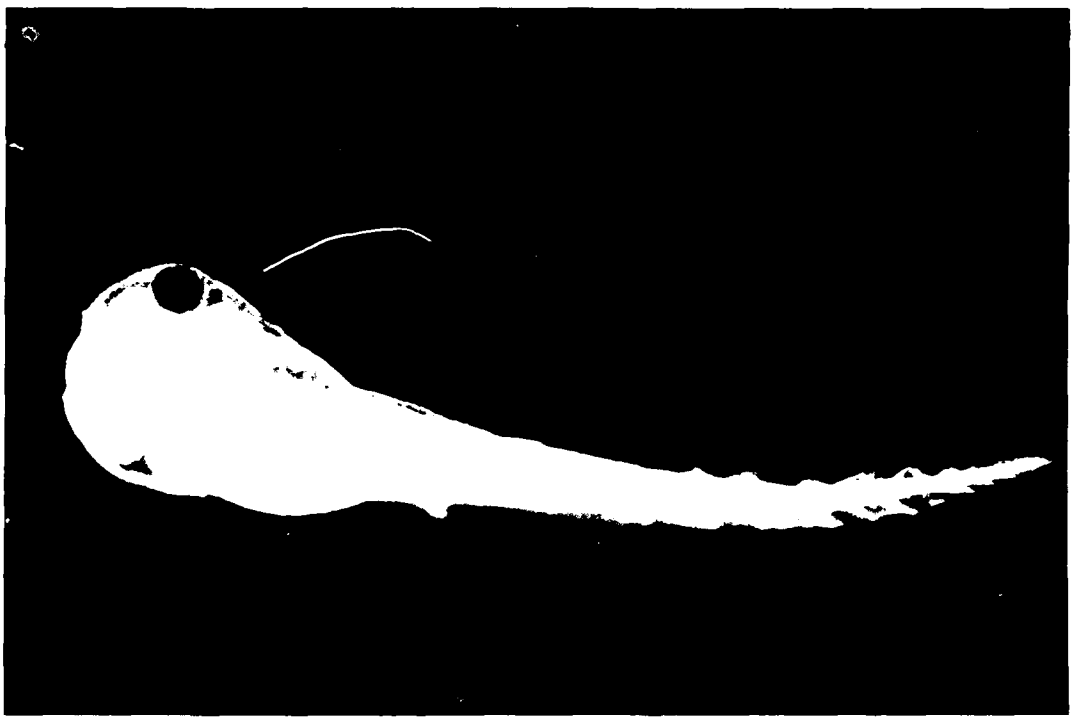

Plate 9B. Effects of a Low Concentration of Triethylene Glycol on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged ventral view presented to show the effect on cardiac and gut region. Embryo exposed to 2% v/v Triethylene glycol.

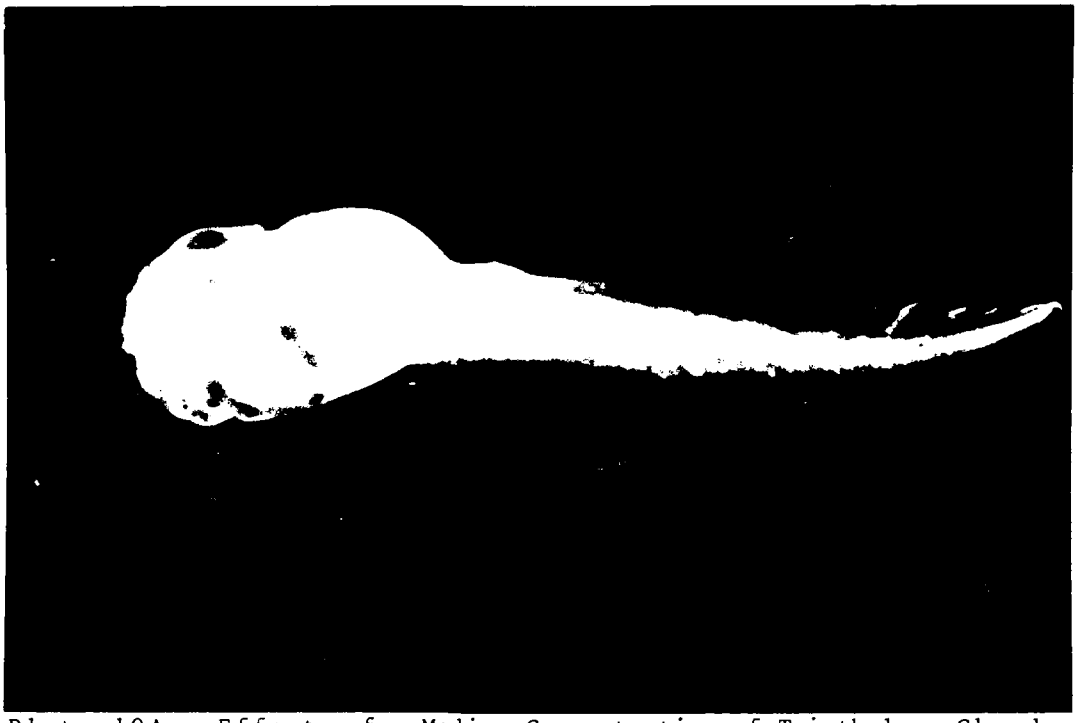

Plate 10A. Effects of a Medium Concentration of Triethylene Glycol on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged ventral view presented to show the effect on cardiac and gut region. Embryo exposed to 2.4 mg/ml Triethylene Glycol.

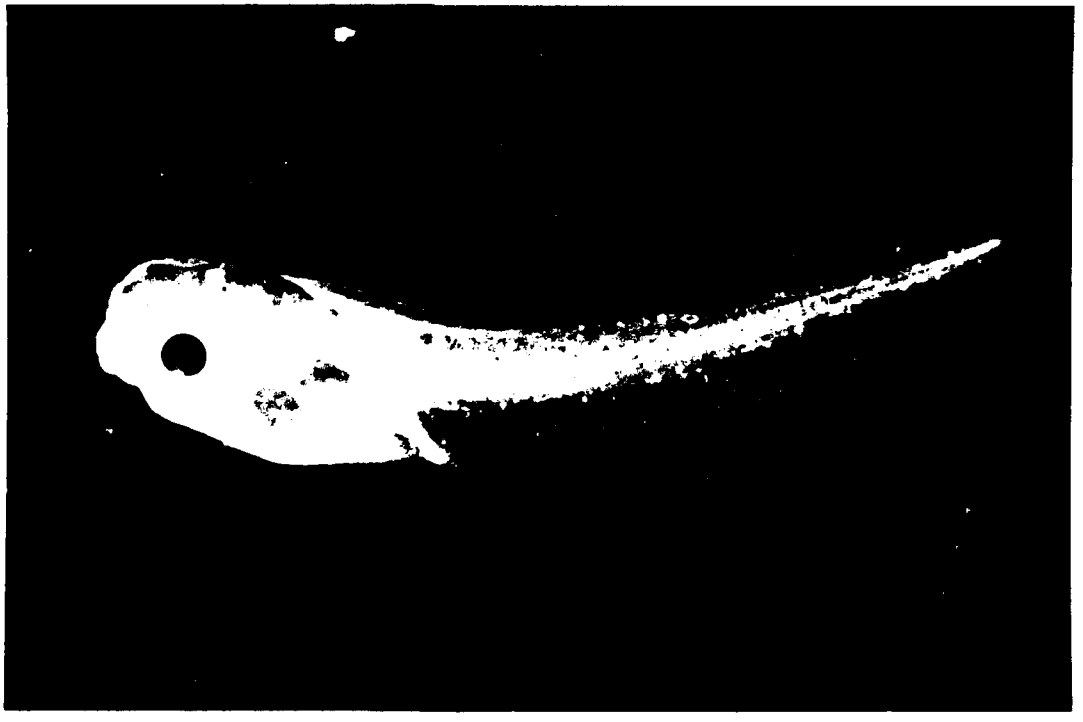

Plate 10B. Effects of a High Concentration of Triethylene Glycol on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 2.7 mg/ml Triethylene Glycol.

Acetone: Acetone can dissolve a large number of organic compounds but it is volatile. Acetone has been shown to potentiate hepatotoxicity induced by Trichloroethylene-carbon tetrachloride mixtures (41). While acetone may be the last solvent of choice, it may prove to be the only solvent that will carry some test compounds into solution. Shepard (34) does not discuss the developmental toxicity of Acetone.

Table 2 shows that the mean TI for Acetone is about 1.7. This is above the cutoff of 1.5 for a nonteratogen. The mean 96-hr LC50 and EC50 (malformation) is 2.19 and 1.29 v/v% respectively and the experiments replicated reasonably well considering the volatility of Acetone (Table 2). Figure 35 shows a representative mortality and malformation dose-response curve for Acetone. Note the obvious separation of the two curves which indicates that Acetone has weak teratogenic potential. The minimum concentration to inhibit growth was 1.25 v/v%. Figure 36 shows a representative growth inhibition curve for Acetone which is flat over a broad concentration range and then increasingly inhibits growth up to the 100% of the 96-hr LC50 level. Even at this concentration, the embryos are only inhibited about 20% of their growth. Plates 11 and 12 show the effect of Acetone on Xenopus development. Plate 11 is a concentration series seen from the side (11A) and ventral (11B) aspects. The embryo from the 0.9 v/v% (second from top) exposure concentration shows essentially no difference from the control (top). This would be near the final acetone solution concentration that would be used if acetone was employed as a carrier solvent. Plate 11 also shows that severe malformations occur only at about 2% concentration and that all organ systems are involved. Plate 12A shows a ventral view of an embryo exposed to 0.9% v/v Acetone. The only apparent change from controls is a slightly reduced gut coiling. This should be regarded not as a malformation but as a case of delayed development. Exposure to clean water and time would probably correct this situation. Plate 12B shows an embryo exposed to 1.5% v/v Acetone and there are indications of stress at this concentration. The choroid fissure of the eye has not yet completely fused at the ventral aspect and there is evidence of edema in the ocular and cardiac regions. These are modest defects, however, considering that 1.5% v/v Acetone is nearly 70% of the 96-hr LC50.

In summary, Acetone is not an ideal solvent because of its high teratogenic index. We have also observed that bacteria can utilize it as a carbon source. However, it may have to be employed for those test compounds that cannot be carried into solution any other way.

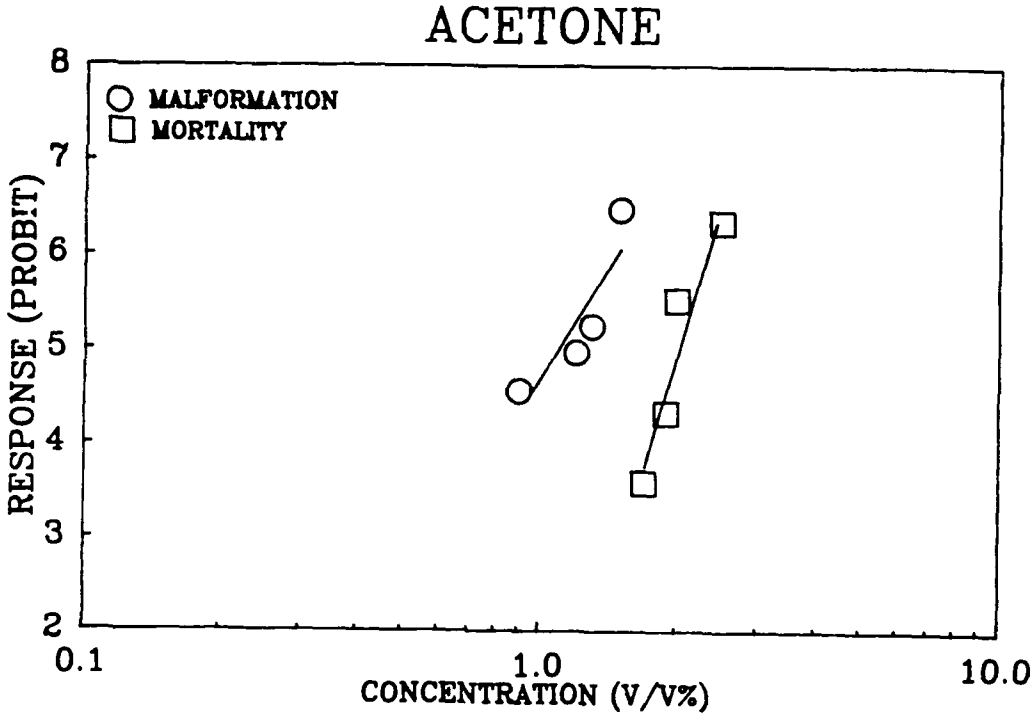

Figure 35. 96-h Mortality and Malformation Dose-Response Curves for Acetone Definitive Test #3. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

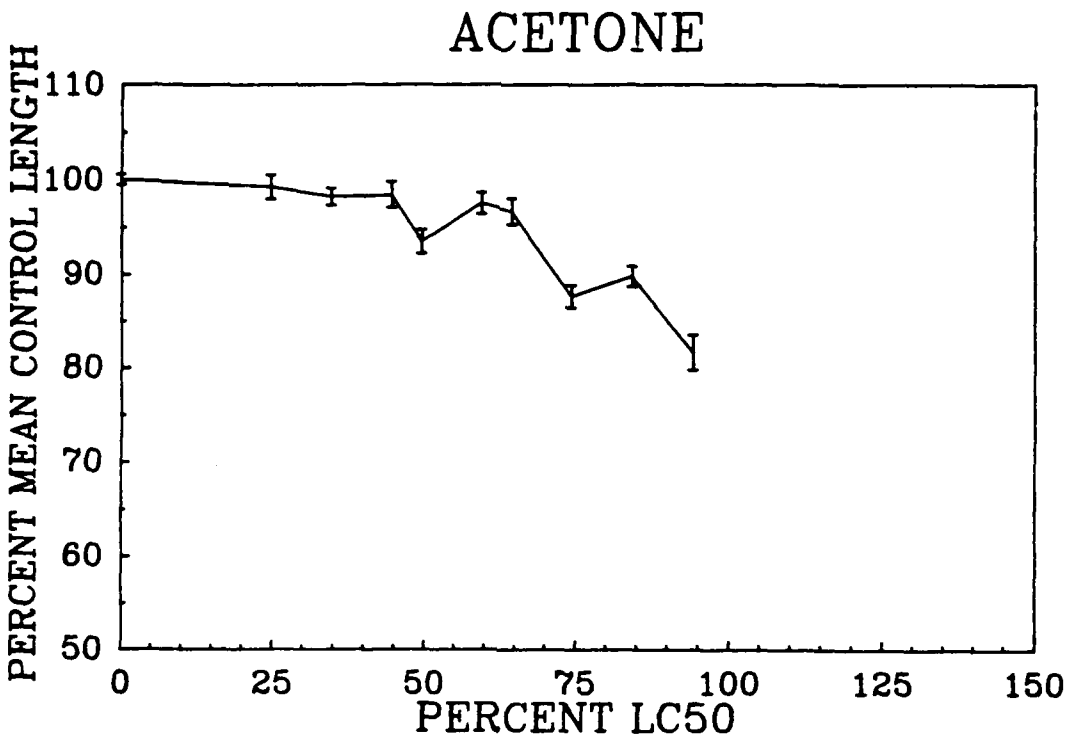

Figure 36. 96-h *Growth Dose-Response Curve* for Acetone Definitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

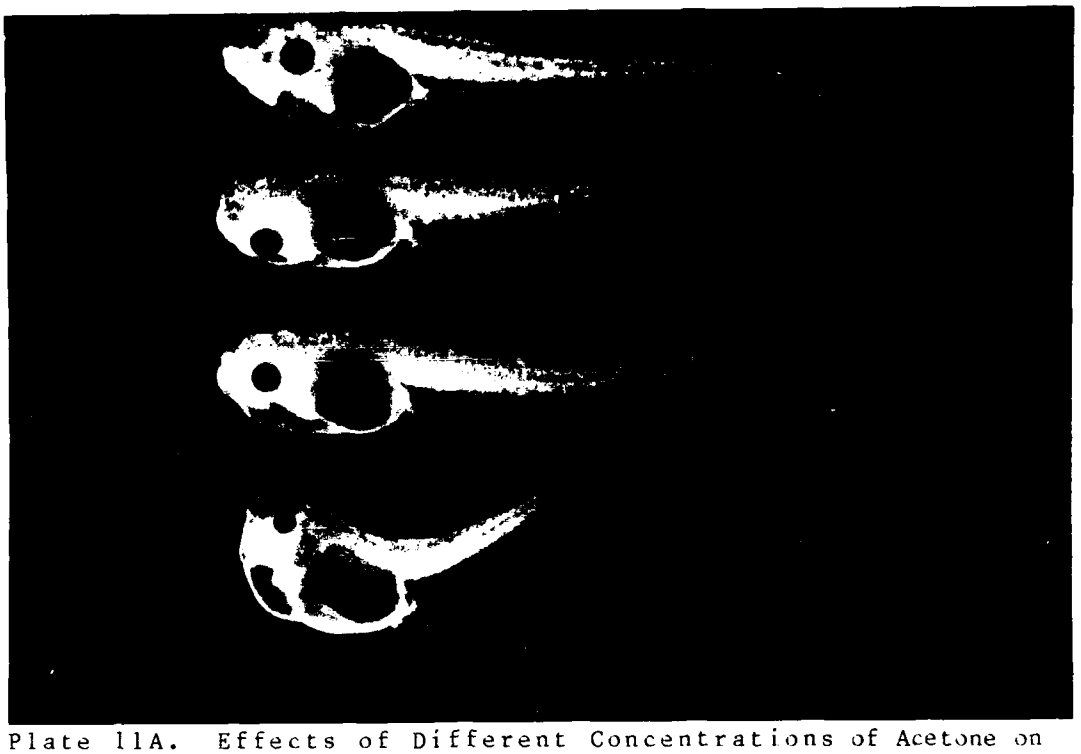

Plate 11A. Effects of Different Concentrations of Acetone on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 0.9% v/v, 1.5% v/v, 2% v/v.

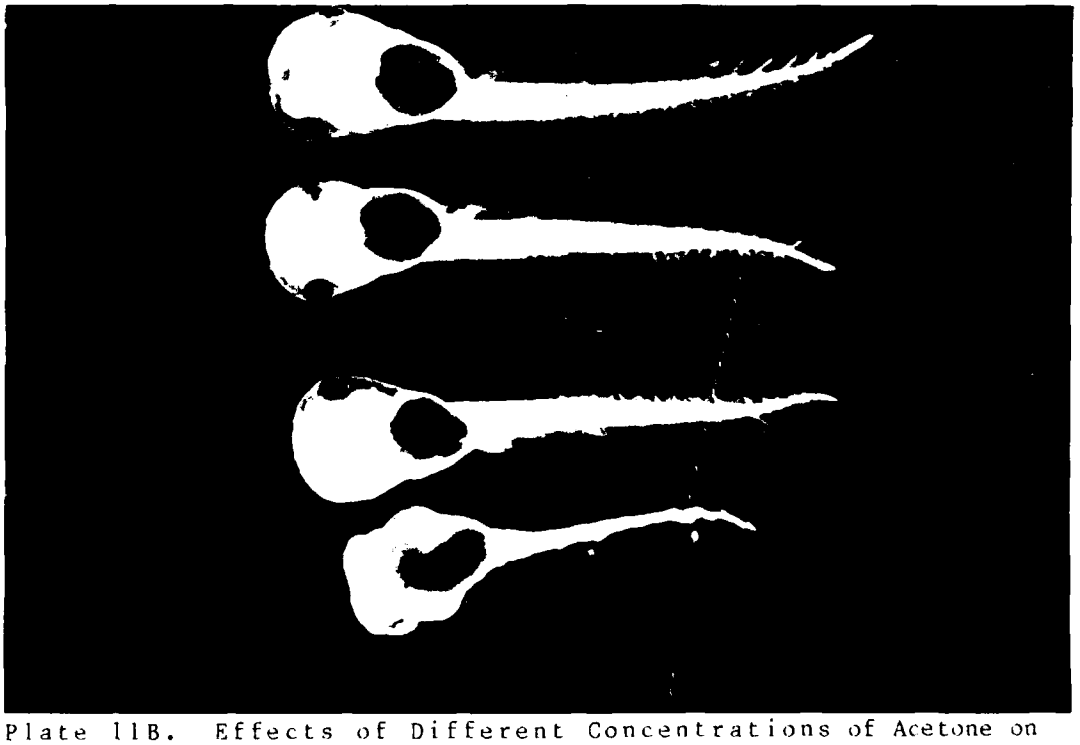

Plate 11B. Effects of Different Concentrations of Acetone on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Ventral view presented to show effect on cardiac and gut regions. From top to bottom: control, 0.9% v/v, 1.5% v/v, 2% v/v.

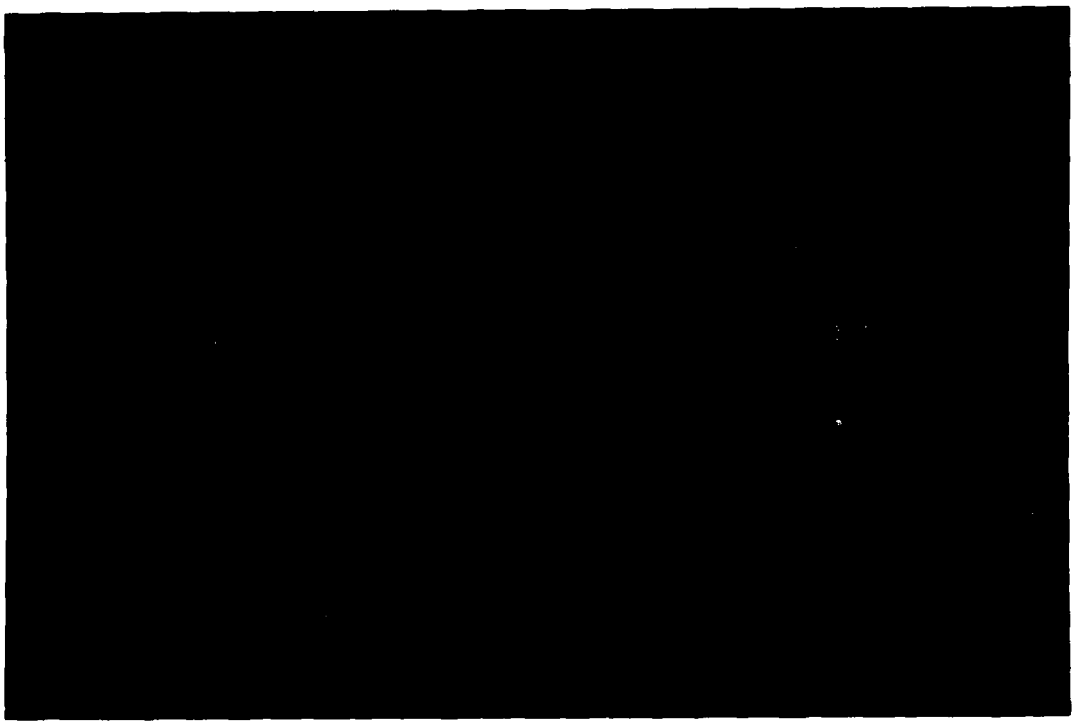

Plate 12A. Effects of a Low Concentration of Acetone on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Ventral view presented to show effect on cardiac and gut regions. Embryo exposed to 0.9% v/v Acetone.

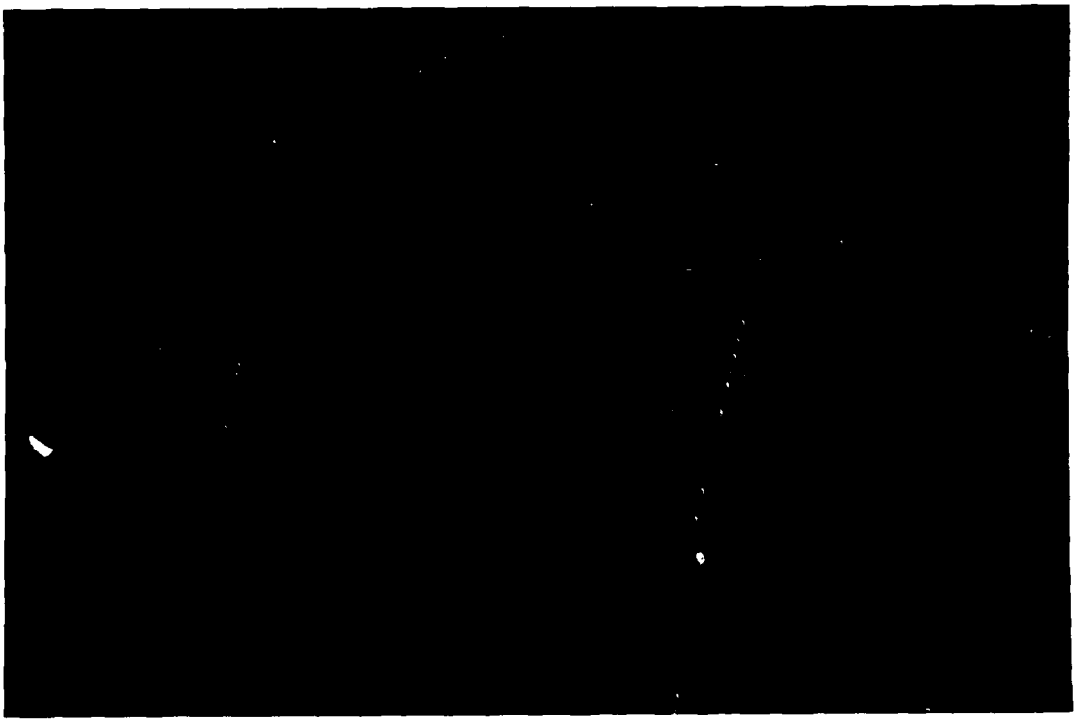

Plate 12B. Effects of a Medium Concentration of Acetone on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 1.5% v/v Acetone.

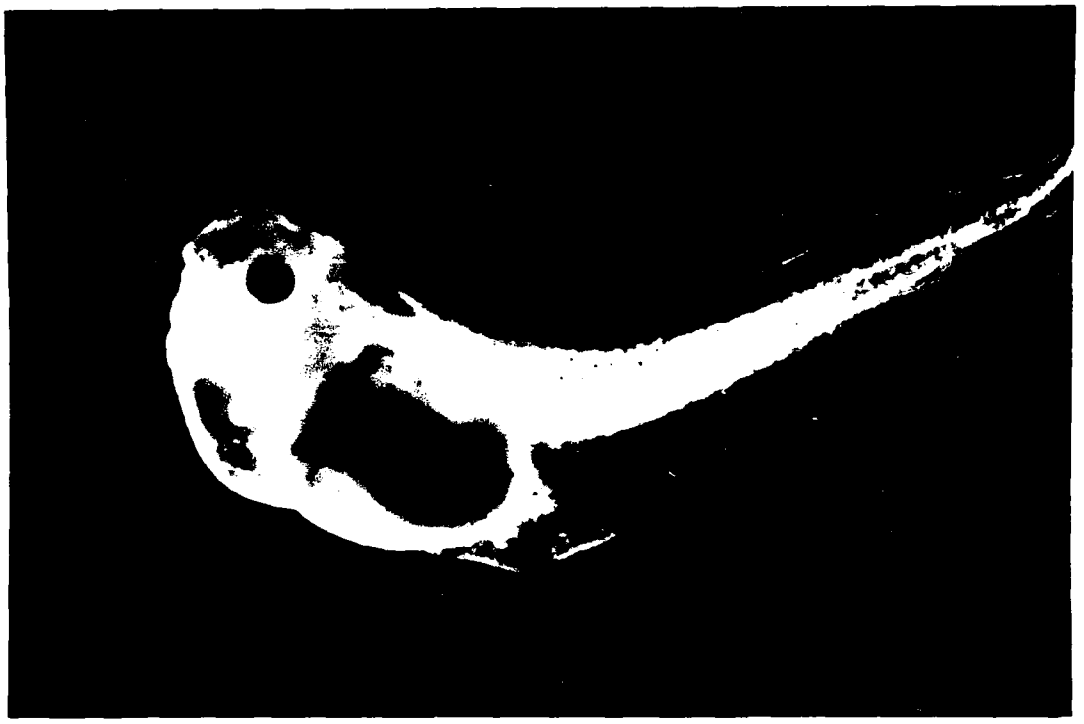

Plate 13. Effects of a High Concentration of Acetone on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 2% v/v Acetone.

Dimethyl Sulfoxide: Dimethyl Sulfoxide (DMSO) is a remarkably good solvent and has relatively low toxicity. DMSO should be listed as a variable negative in terms of its developmental toxicity (34). Caujolle et al. (42) reported teratogenic responses in rabbits, chickens, mice and rats when DMSO was administered at very high doses. However, at low doses few defects were seen. Juma and Staples (43) found increased resorptions in the rat embryo at high dose levels suggesting that DMSO is primarily embryotoxic. These variable results reported in the literature may be due to differences in dosage in the various studies. High doses of even nonteratogens can cause abnormal development in the few surviving offspring.

Table 2 shows the results of three definitive experiments on the effect of DMSO on Xenopus embryos. The mean TI was 1.4 while the mean 96-hr LC50 and EC50(malformation) was 1.81 and 1.31% v/v respectively. Dumont reported a TI of 1.75 for DMSO so our values are not too far apart. The mean minimum concentration that inhibits growth was 1.38% v/v. There was excellent agreement among all three experiments. The results prove that a 1% v/v final concentration of DMSO could successfully be used as a carrier in FETAX (This concentration is safely below the MCIG). Figures 37 and 39 show the dose-response curves from the latter two definitive experiments listed in Table 2. The mortality and malformation curves are generally close together indicating low teratogenicity. There are a number of data points between the 0 and 100% effect levels for both endpoints and the data fit is close to the line. The NOEC is around 1.1 to 1.2% v/v for both endpoints. Figures 38 and and 40 show the effects of DMSO on Xenopus growth. Figure 38 shows an indication of hormesis as embryos were longer after DMSO exposure than controls. This phenomenon was not borne out in Figure 40. Both Figures show that growth inhibition does not become a serious factor until well past 50% of the 96-hr LC50 concentration. After this point a severe reduction in growth is observed. Plates 14 and 15 show the typical effects of DMSO on Xenopus embryos after 96 hr of continuous exposure. Plate 14A shows a progression of increasing malformation and growth retardation in increasing concentrations of DMSO. There is little spinal kinking and edema. Facial, head and eye malformations occur at higher concentrations of DMSO. At 1.3% v/v DMSO, the effects are slight (Plates 14A &B). A reduction in embryo size and gut coiling are seen. At 1.7% v/v DMSO, there is evidence of some edema as well as problems in gut coiling and development of the facial region (Plate 15A). At 2% v/v DMSO (Plate 15B), the embryo is severely malformed with all major organ systems being involved.

In summary, DMSO is an excellent solvent and exhibits low teratogenicity in FETAX. There should be no problem in using 1% v/v final concentrations of DMSO as a carrier unless further studies show real interaction problems.

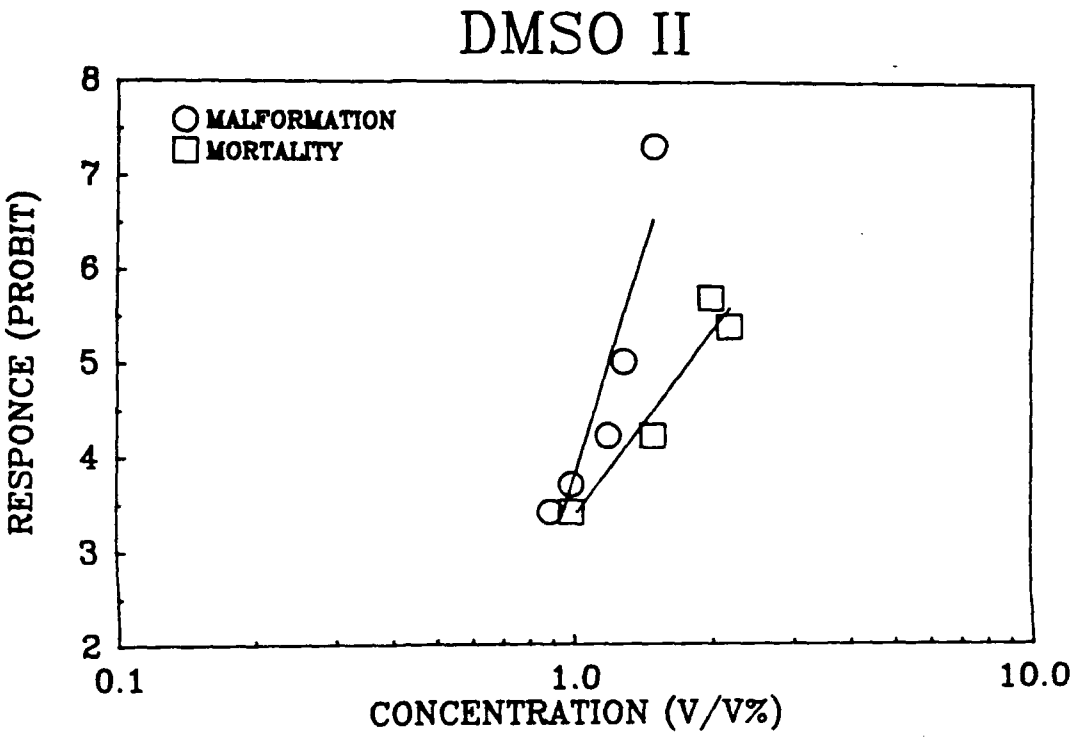

Figure 37. 96-h Mortality and Malformation Dose-Response Curves for Dimethyl Sulfoxide Definitive Test #2. The curve includes only those points used in producing the dose-response curves although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

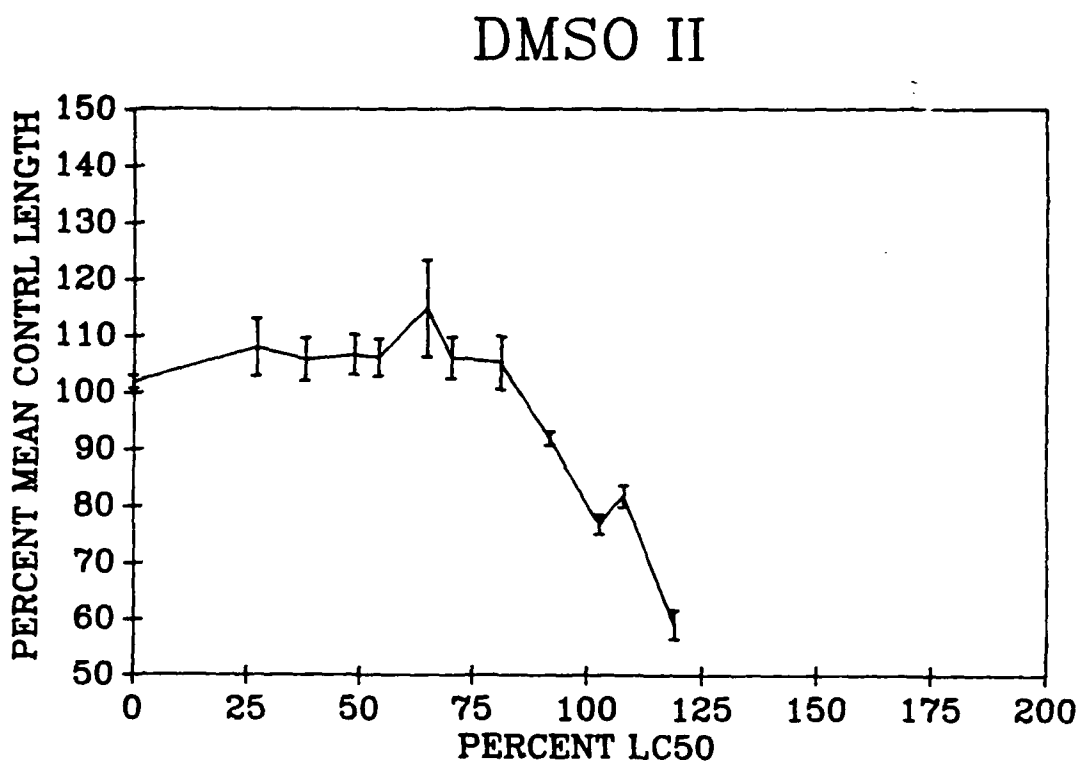

Figure 38. 96-h Growth Dose-Response Curve for Dimethyl Sulfoxide Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

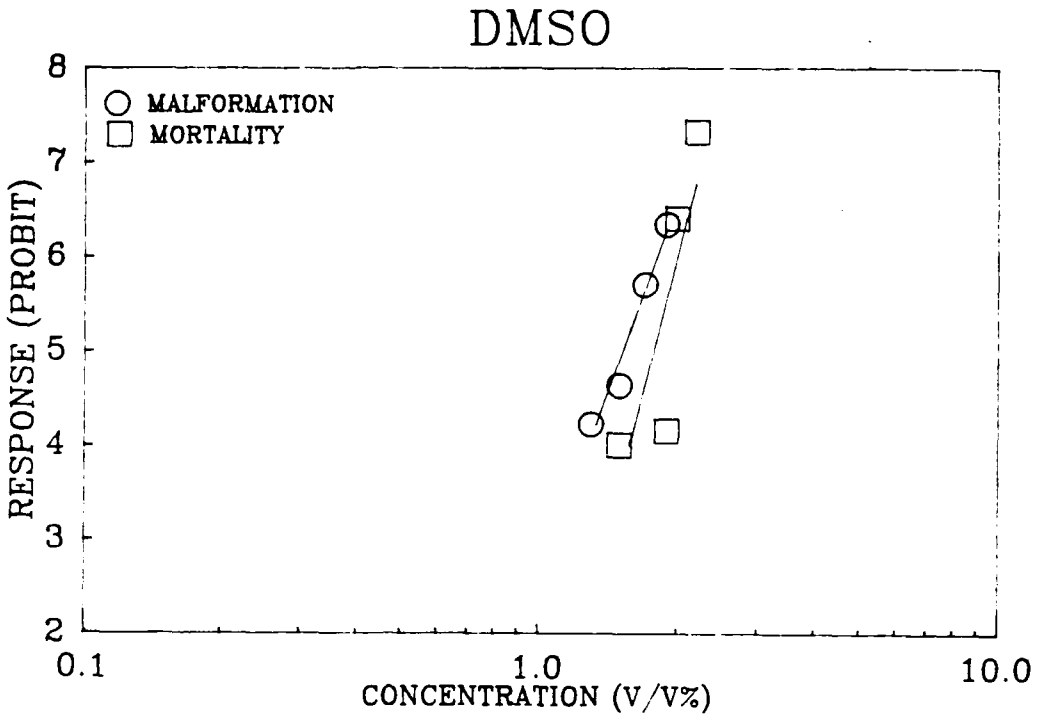

Figure 39. 96-h Mortality and Malformation Dose-Response Curves for Dimethyl Sulfoxide Definitive Test #3. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

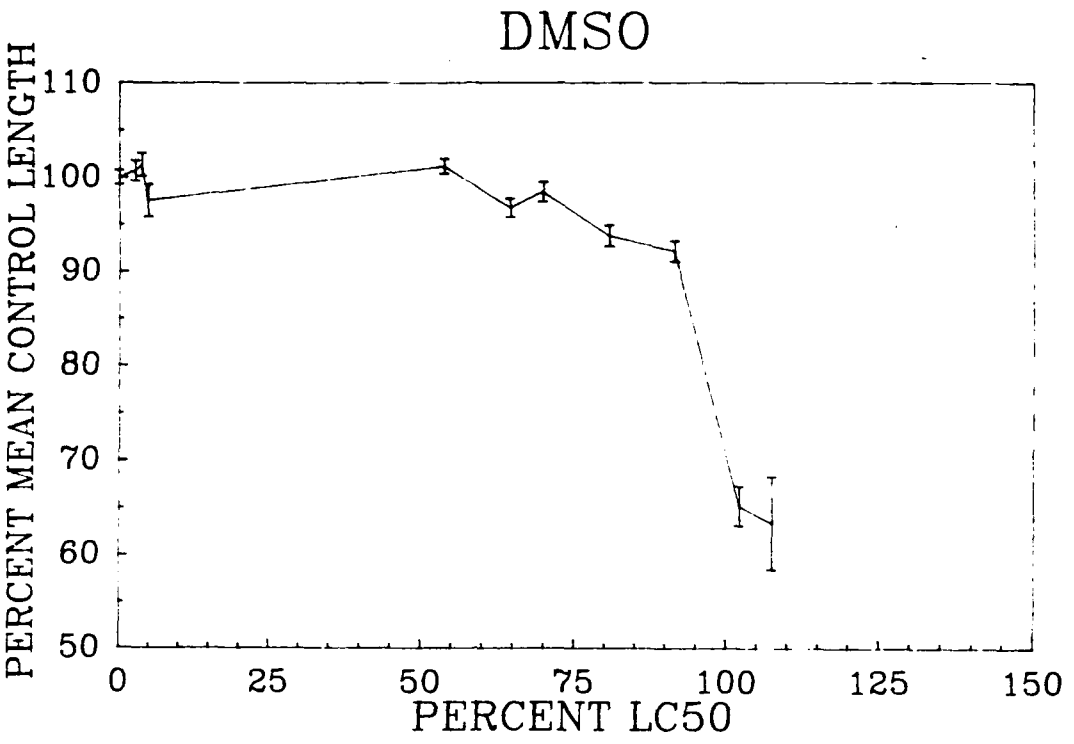

Figure 40. 96-h Growth Dose-Response Curve for Dimethyl Sulfoxide Definitive Test #3. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

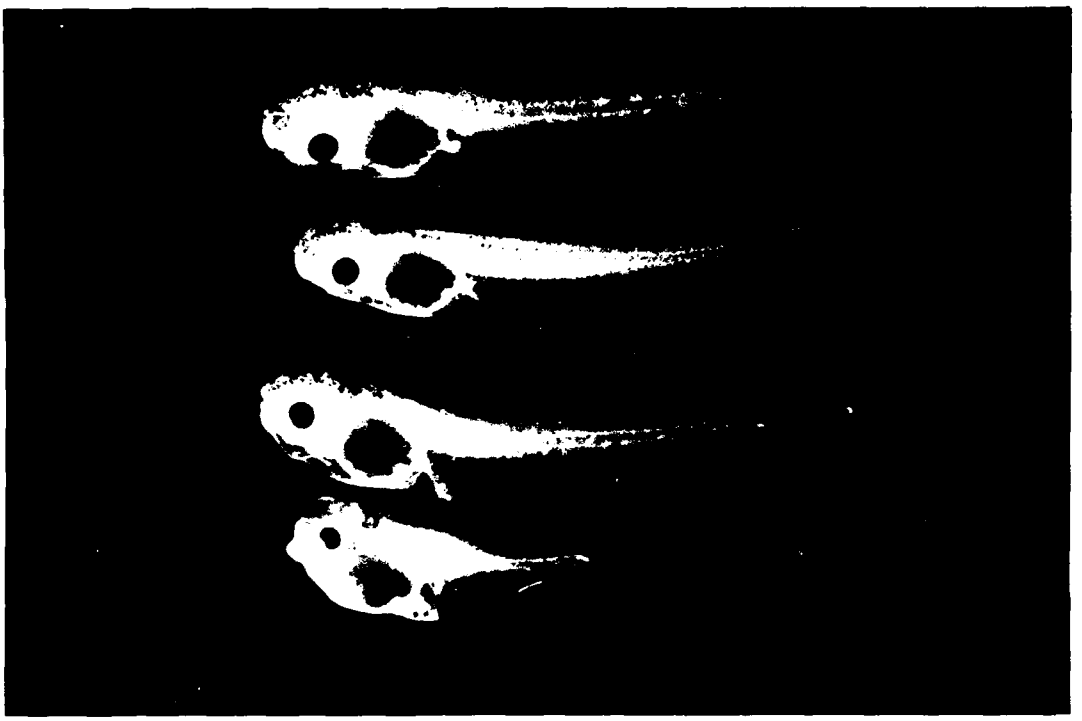

Plate 14A. Effects of Different Concentrations of Dimethyl Sulfoxide on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 1.3% v/v, 1.7% v/v, 2% v/v.

Plate 14B. Effects of a Low Concentration of Dimethyl Sulfoxide on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged ventral view presented to show the effect on cardiac and gut region. Embryo exposed to 1.3% v/v Dimethyl Sulfoxide .

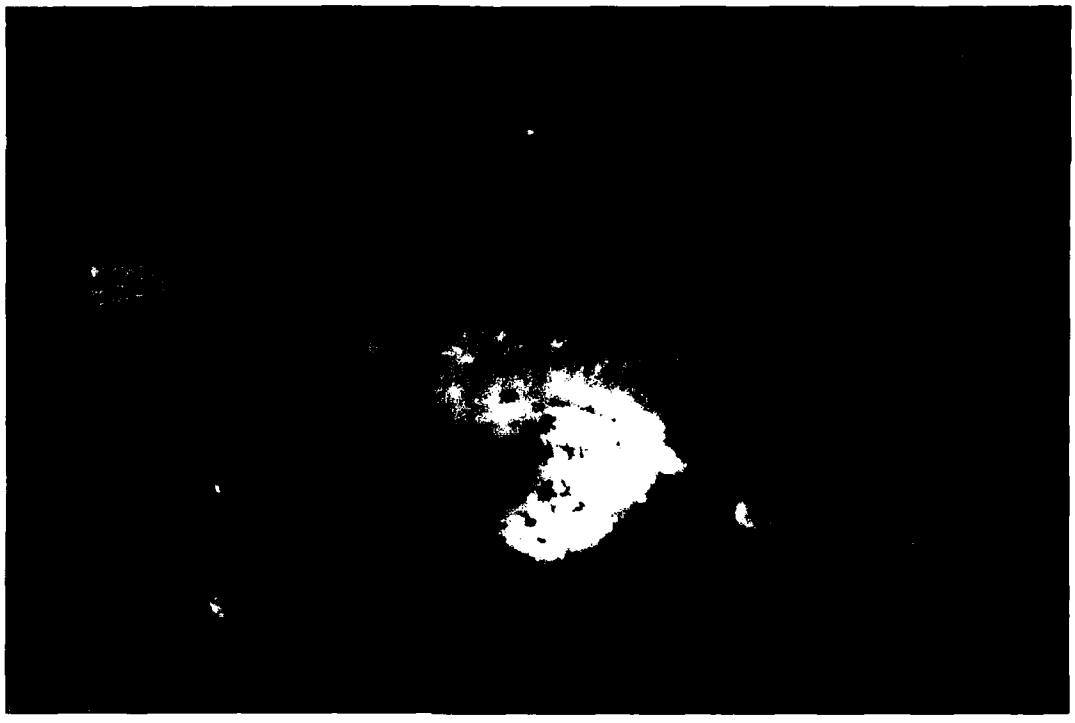

Plate 15A. Effects of a Medium Concentration of Dimethyl Sulfoxide on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 1.7% v/v Dimethyl Sulfoxide.

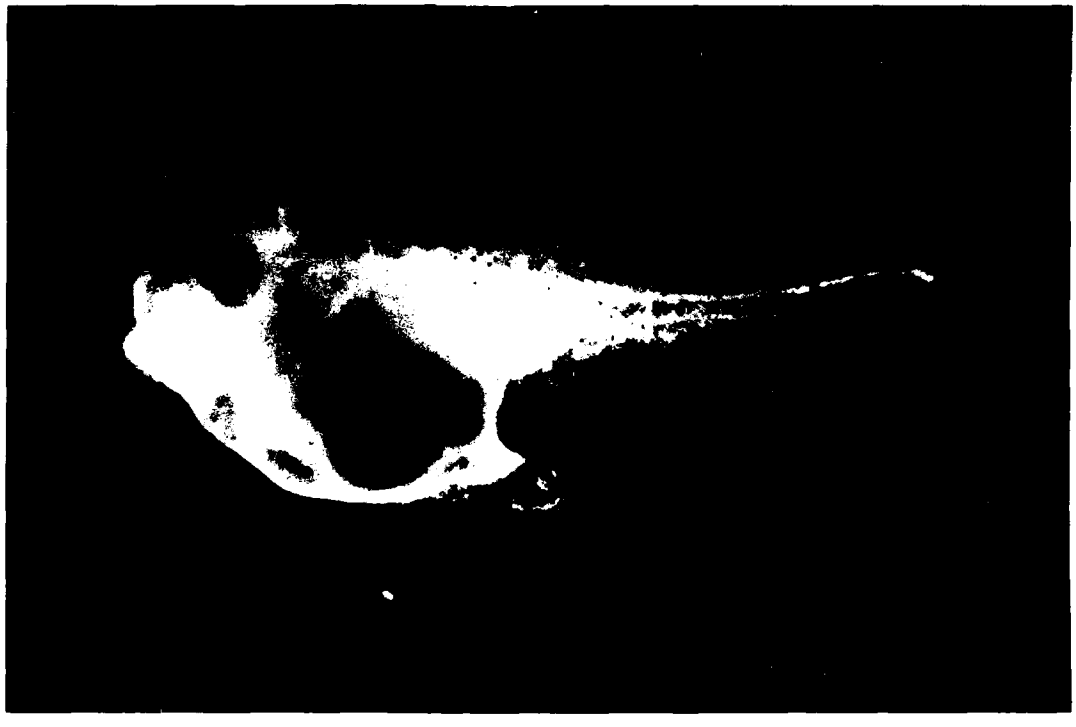

Plate 15B. Effects of a High Concentration of Dimethyl Sulfoxide on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 2% v/v Dimethyl Sulfoxide.

Trans-retinoic acid: Work on this compound is being supported by the Oklahoma Center for the Advancement of Science and Technology (OCAST). The data is presented here in order to present a clear idea as to the nature of the upcoming solvent interaction study. trans-retinoic acid is a classic teratogen with numerous listings in Shepard (34). It is listed by Smith et al. (22) as a strong teratogen that is quite insoluble in water. Despite its insolubility in water, enough of the compound gets into solution to have an effect in FETAX without the use of a carrier. This made it an ideal compound to use in the carrier interaction study because results of retinoic acid alone vs retinoic acid plus carrier could be easily compared. Table 2 shows that three different teratogens (Trans-retinoic acid, Me-mercury chloride and 6-Aminonicotinomide) of differing solubilities and teratogenic actions were selected for this study as well as Trichloroethylene, a nonteratogen, of low water solubility.

Table 2 shows that there was difficulty in repeating the dose-response tests for Trans-retinoic acid. The mean TI was close at 10.5 but that in the first definitive experiment the 96-hr LC50 was only 0.246 ug/ml versus 0.5 for the second definitive. Upon inspection of the data in Table 2 both the 96-hr LC50 and EC50(malformation) of the first test were approximately 2 fold lower than the second test. Although we checked the concentrations of each stock solution spectrophotometrically, it is possible an error was made in weighing or dissolving the insoluble Trans-retinoic acid. Previous range tests also showed this type of variability for this compound. The high TI of 10.5 proved that Trans-retinoic acid was a strong teratogen in FETAX and this result agrees well with the mammalian literature. Sabourin and Faulk (14) obtained a TI >2.6 for Trans-retinoic acid while Dumont obtained a TI of 6.6. It should be remembered that Sabourin and Faulk only reported ranges of TI and not actual values. Figures 41 and 43 show the mortality and malformation dose-response curves. They are widely separated, have essentially the same slope and there are a good number of data points that make up each curve. The mean MCIG is 0.07 which is 28% of the 96-hr LC50. From Figure 42 and 44 is can be seen that growth inhibition increases rapidly faster about 25% of the 96-hr LC50. The maximum amount of growth inhibition is about 35% (65% of control length). Thus, the growth inhibition data is supportive of the hypothesis that Trans-retinoic acid is a teratogen. Plates 16 and 17 show the effects of increasing Trans-retinoic acid concentrations on Xenopus development (See 16A for overview). Plate 16B shows the effect of dose near the EC50(malformation) had on development. Minor malformations were seen in the face, eye and gut region. At 0.2 ug/ml severe malformations were seen that involve all major organ systems (Plate 17A). Curiously, The tail was not kinked. At very high concentrations (0.5 ug/ml), very severe malformations were observed. This embryo was alive at 96 hr but severely malformed. The eye is cyclopic and the gut is nearly straight. At this concentration, nearly half of the embryos were still alive.

Despite the variability in results, Trans-retinoic acid is an ideal compound with which to test the possibility of solvent interactions on Xenopus development. The question arises as to whether the same defects will be seen if there is interaction or whether new malformations will be caused.

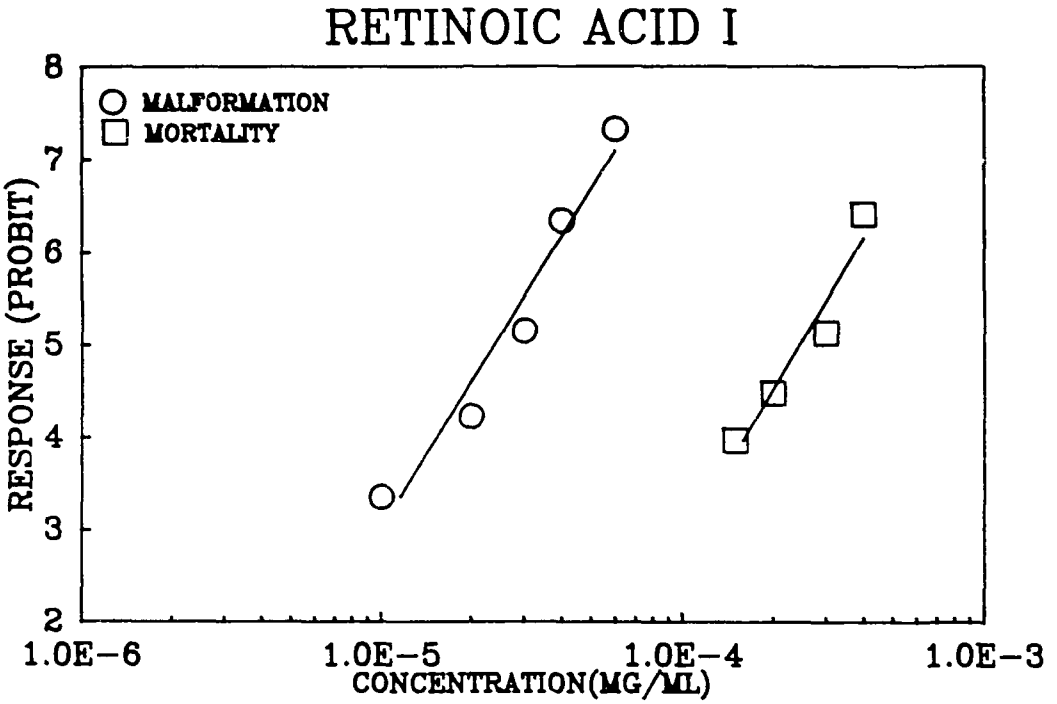

Figure 41. 96-h Mortality and Malformation Dose-Response Curves for Retinoic Acid, Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

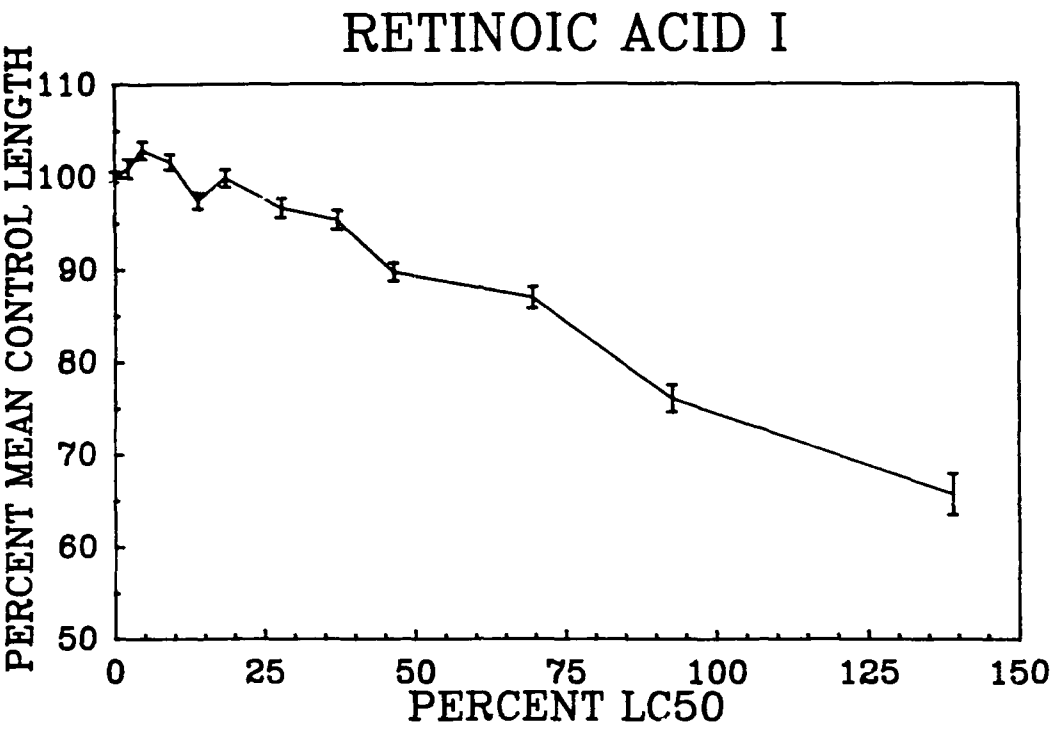

Figure 42. 96-h Growth Dose-Response Curve for Retinoic Acid, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

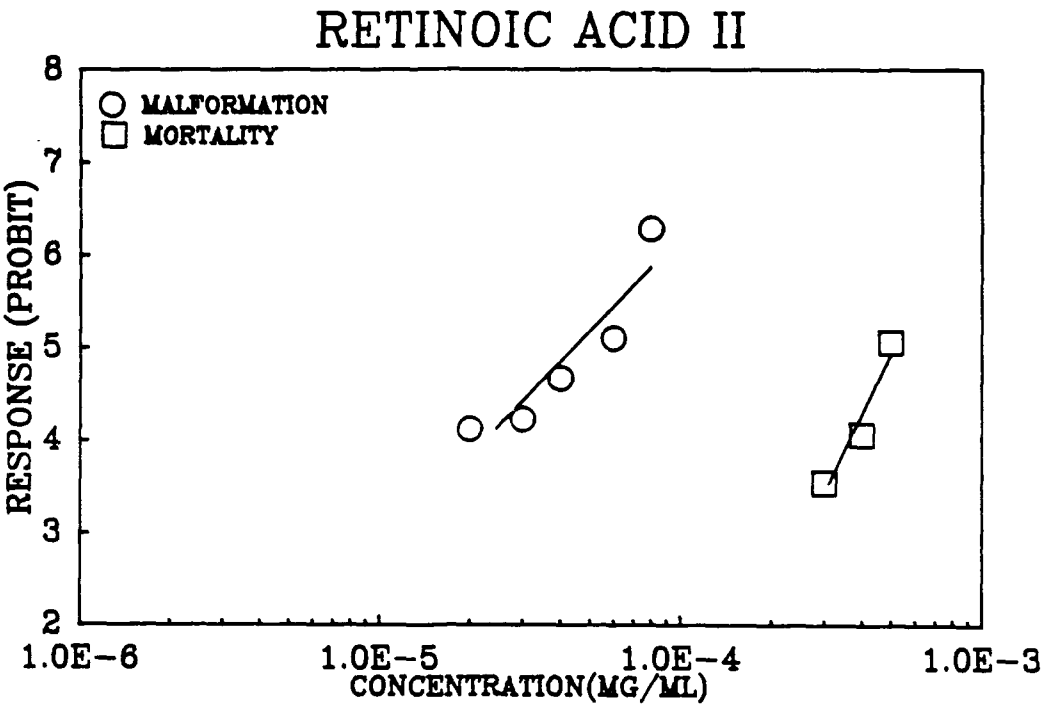

Figure 43. 96-h Mortality and Malformation Dose-Response Curves for Retinoic Acid, Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

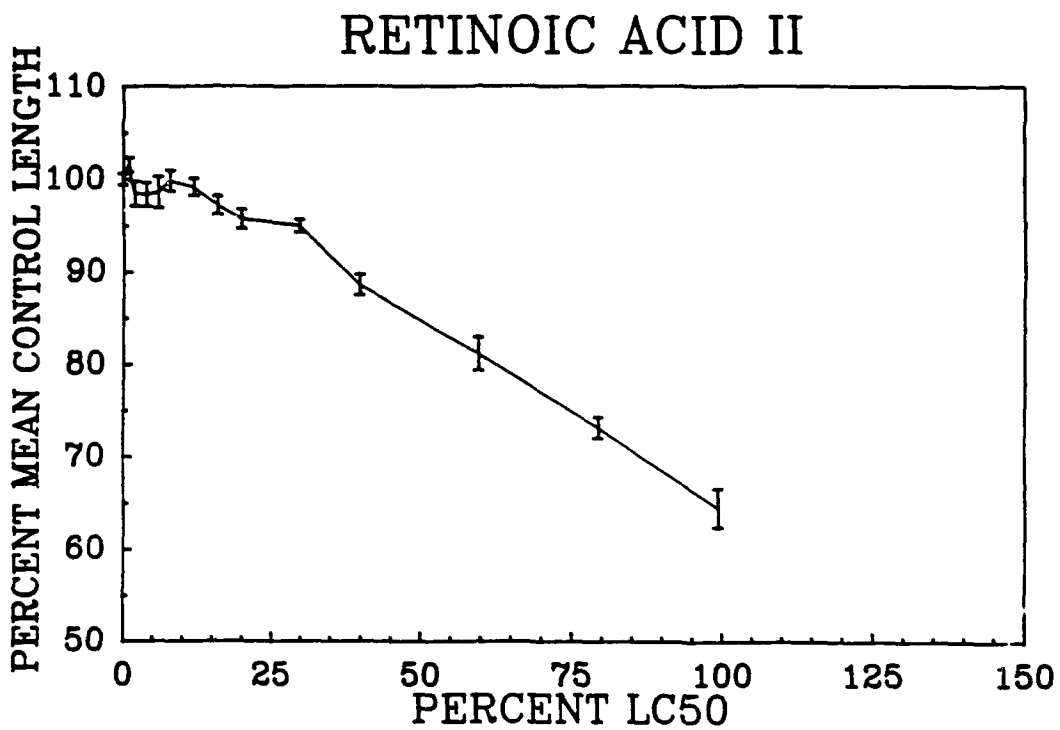

Figure 44. 96-h Growth Dose-Response Curve for Retinoic Acid, Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

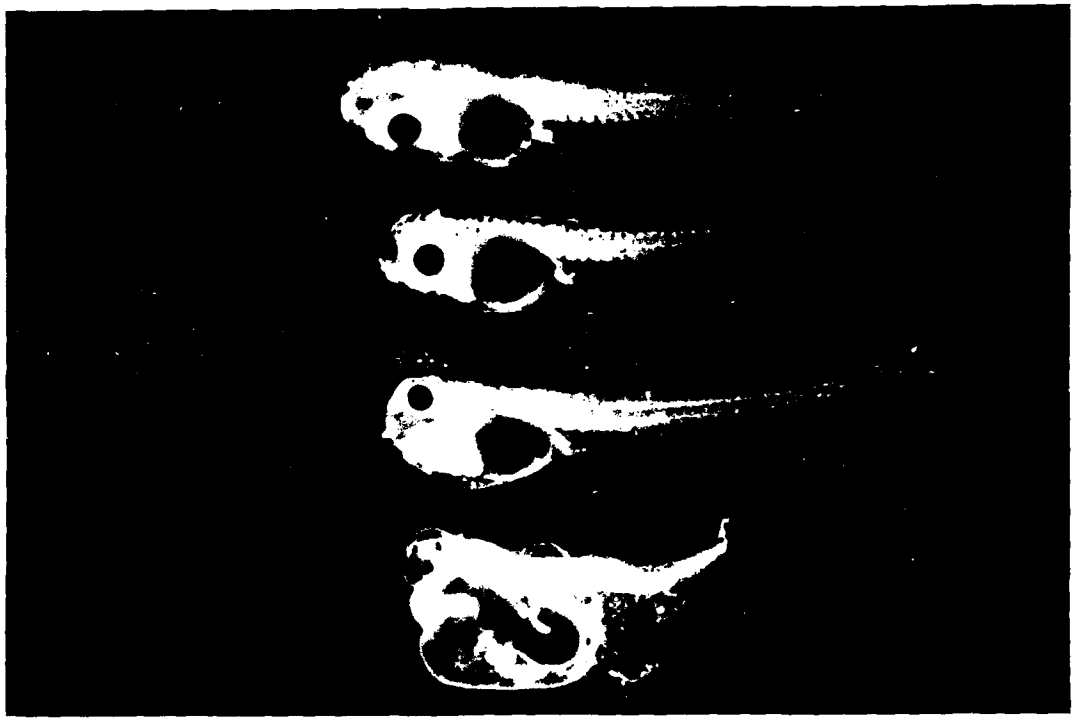

Plate 16A. Effects of Different Concentrations of Trans-retinoic acid on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 0.02 ug/ml, 0.2 ug/ml, 0.5 ug/ml.

Plate 16B. Effects of a Low Concentration of Trans-retinoic acid on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.02 ug/ml Trans-retinoic acid.

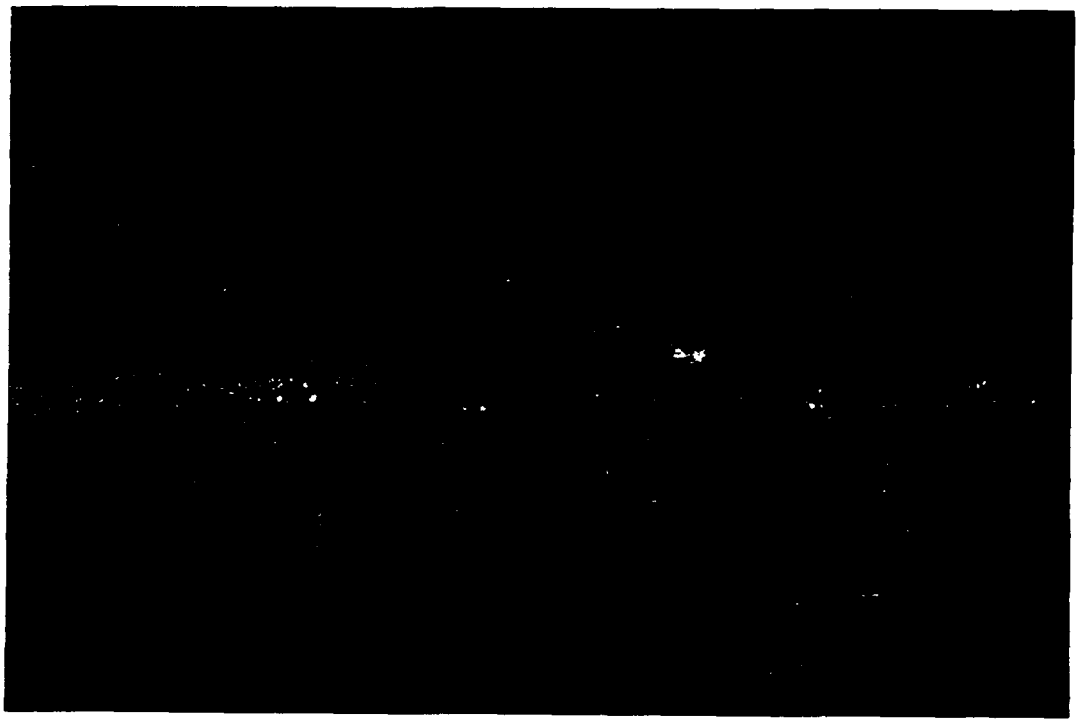

Plate 17A. Effects of a Medium Concentration of Trans-retinoic acid on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.2 ug/ml Trans-retinoic acid.

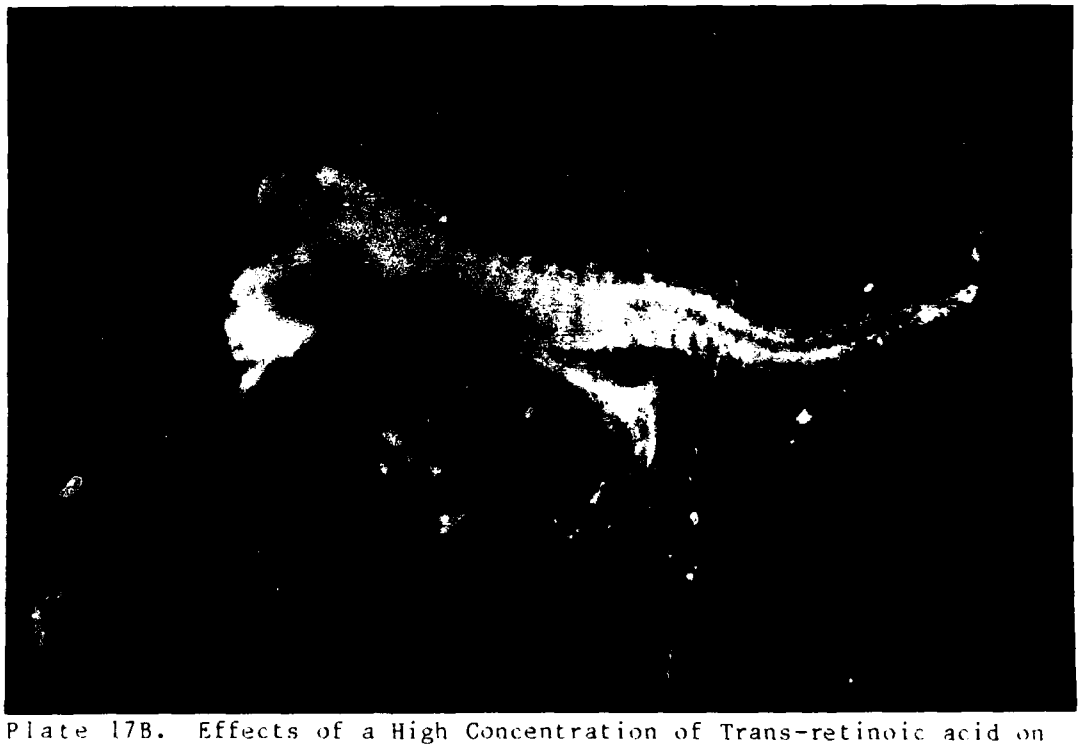

Plate 17B. Effects of a High Concentration of Trans-retinoic acid on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Enlarged side view presented to show the effect on brain and eye region. Embryo exposed to 0.5 ug/ml Trans-retinoic acid.

3. Metabolic Activation System

Acetaminophen: Acetaminophen (Tylenol) is listed as a variable negative that may be altered by metabolism (Table 3). Shepard (34) indicated that there was no evidence for human teratogenicity and most of the animal studies were negative. Potter et al. (44) presented evidence that cytochrome P-450 was involved in the conversion of Acetaminophen to an N-hydroxy derivative that causes hepatic necrosis in rats. This made the study of Acetaminophen interesting because it may be negative without an in vitro metabolic activation system (MAS) but positive with the rat liver MAS. Mammals may be able to ameliorate these negative effects because of the placental relationships.

Table 2 shows the effects of Acetaminophen on Xenopus growth and development. This compound has turned out to be very unusual in its mode of action. Although its solubility in aqueous solutions is excellent it nonetheless gave highly variable results. We have run this test 6 times with three different operators and continue to get unusual results. We only presented three of these experiments to conserve space. Dose-response curves undulate slightly (Figure 45) and the 96-hr LC50 can shift as is seen in the third definitive test with antibiotics. Some of the variability seen can be due to the presence of bacteria as the inclusion of penicillin and streptomycin improve embryo survival (definitive #3). However, interaction between antibiotic and Acetaminophen must be considered. We are continuing to explore this possibility in our lab. Acetaminophen is negative in FETAX for at least the majority of the tests conducted as the mean TI for definitives 1 and 2 is only 1.3. The mean MCIG is 0.11 and Figures 46 and 48 show that Acetaminophen does not affect growth until nearly 65% of the 96-hr LC50. Therefore, the best data available shows that Acetaminophen is a negative in FETAX when there is no metabolic activation system present. At this time we have not added the MAS to the experiment. If the mammalian literature is correct, the addition of rat liver microsomes may affect the 96-hr LC and EC50(malformation) but this should not change the TI or the final conclusion that Acetaminophen is not a developmental toxicant. At this time we do not have any pictures of Acetaminophen treated embryos.

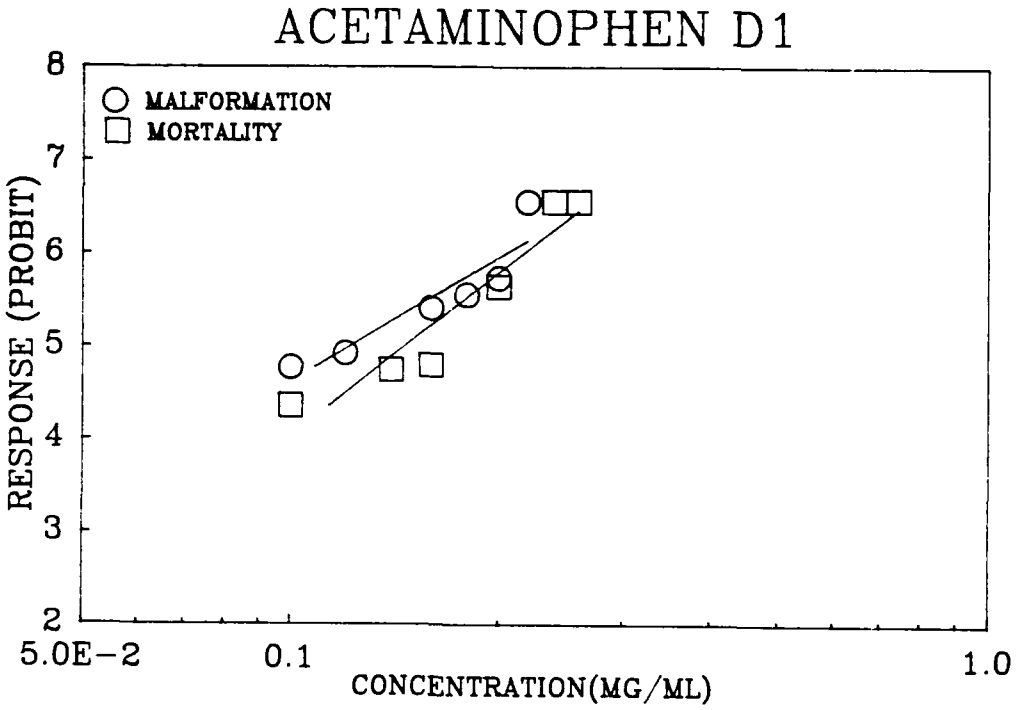

Figure 45. 96-h Mortality and Malformation Dose-Response Curves for Acetaminophen, Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

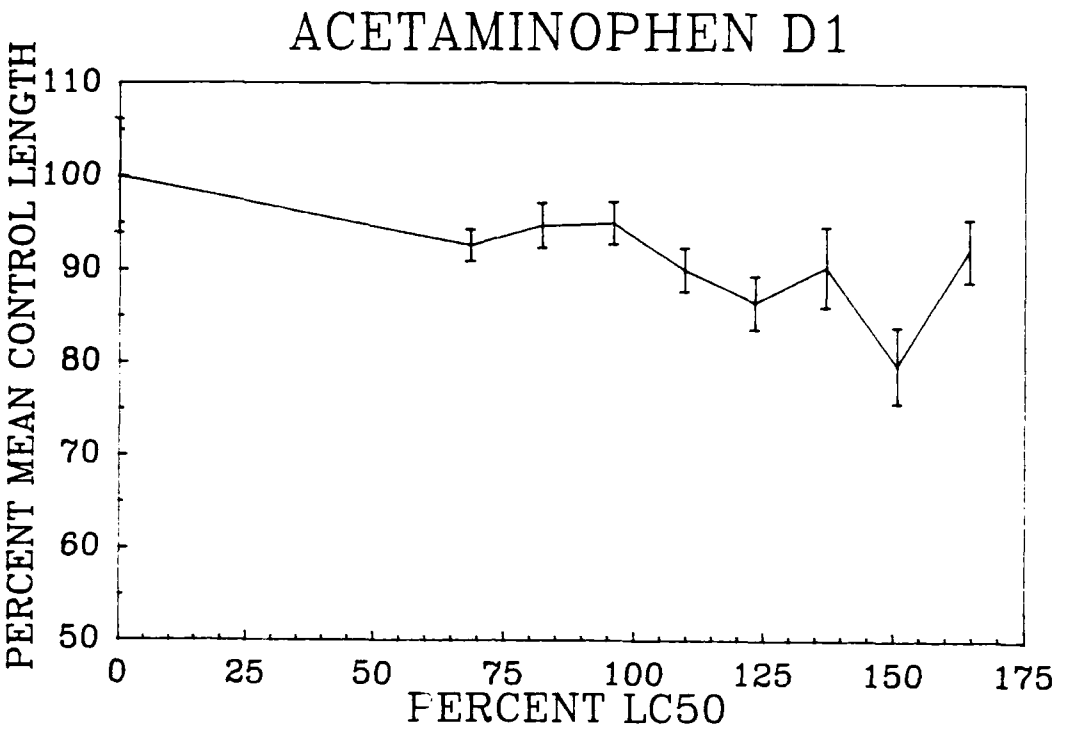

Figure 46. 96-h Growth Dose-Response Curve for Acetaminophen, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

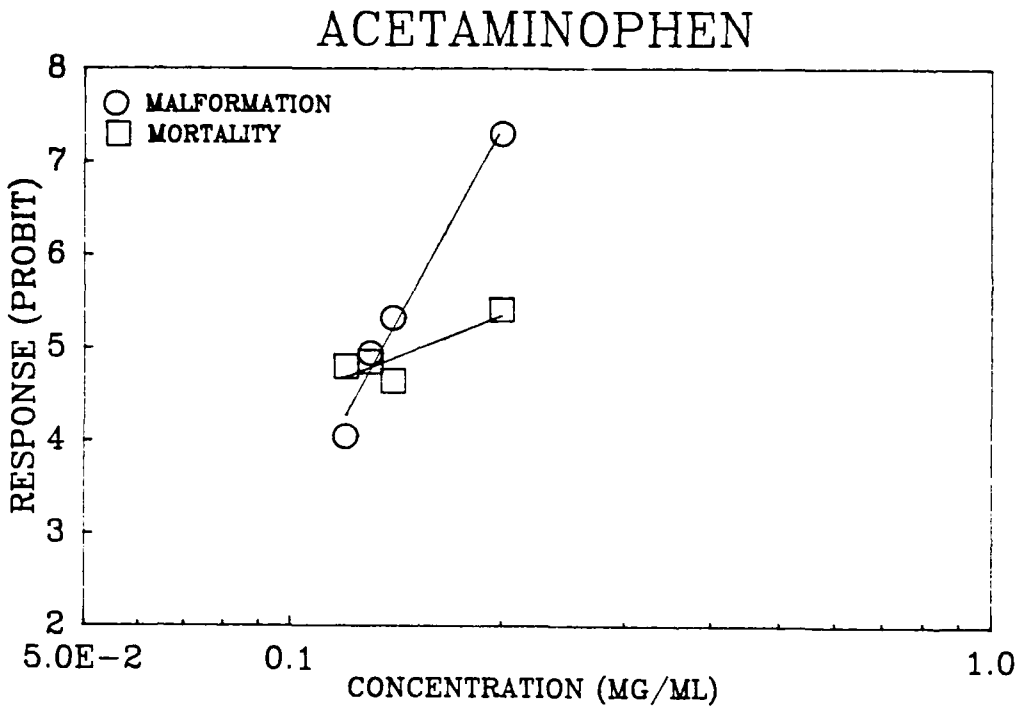

Figure 47. 96-h Mortality and Malformation Dose-Response Curves for Acetaminophen, Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

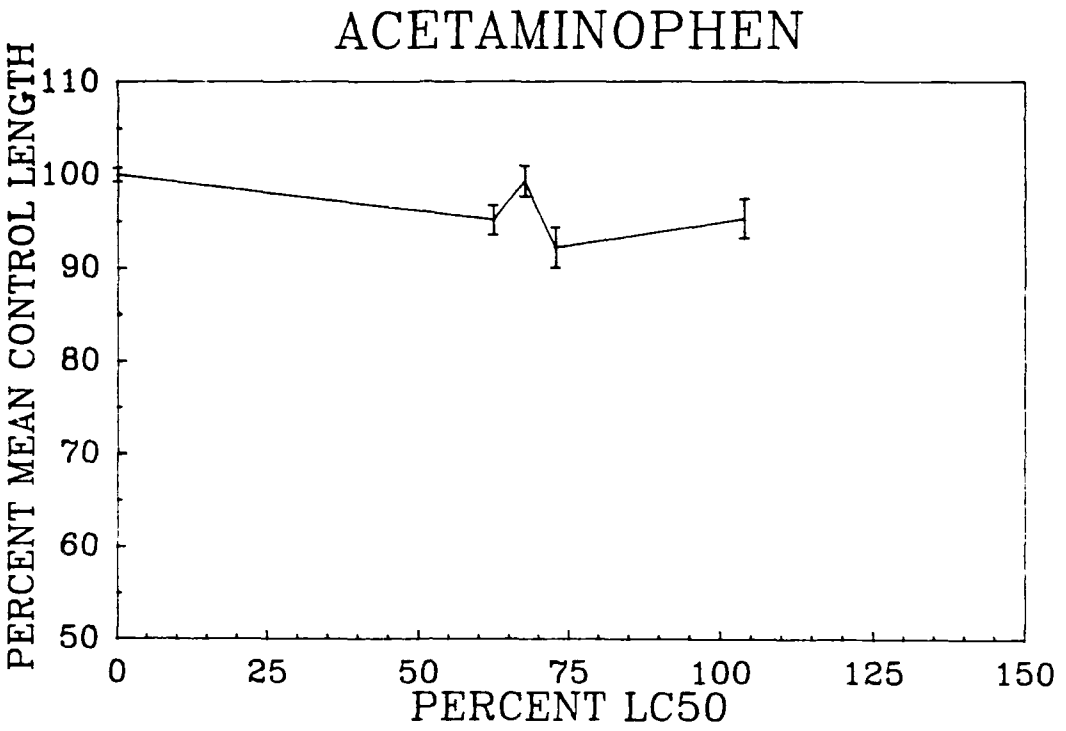

Figure 48. 96-h Growth Dose-Response Curve for Acetaminophen, Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

Acetazolamide: We have only run a single range test on Acetazolamide to date (Table 3). Shepard (34) has 9 listings for Acetazolamide many of which indicate that Acetazolamide has significant developmental toxicity. Smith et al (22) provide evidence that Acetazolamide is a teratogen in rodents but not rabbits and monkeys. They also indicate that it is not metabolized. For this reason we listed it in Table 3 as a variable positive that was not subject to MAS. Sabourin and Faulk (14) reported a TI of <1.6 for Acetozolamide. However, Dumont obtained a TI of 3.46 for this compound. We have only performed a single range test without MAS to date and we used concentrations that were too low to observe an effect.

We have scheduled further work on this compound for the last quarter of the project.

Benzo(a)pyrene: Benzo(a)pyrene (BP) is a common environmental contaminant which is often produced through combustion. Shepard (34) lists BP as a developmental toxicant with more resorptions (embryolethality) occurring at higher concentrations than malformations. There is ample evidence that metabolic activation plays a crucial role in the conversion of BAP to an active metabolic that is mutagenic and able to bind to macromolecules such as DNA (45). We have listed BAP as a positive teratogen that requires metabolic activation for its activity (Table 3). The mean TI without MAS is listed in Table 3 as >1 but the value is really not able to be calculated because we could not get enough into solution to get an adequate 96-LC50. The mean 96-hr EC50(malformation) was 10 ug/ml without MAS. With the in vitro MAS, it was still not possible to get an 96-hr LC50 because of solubility problems but the 96 hr-EC50(malformation) was lowered to 1.17 ug/ml thereby increasing the spread between the theoretical mortality curve and the malformation dose-response curve. Figures 49 and 51 show two typical malformation dose-response curves for BP with and without MAS. It can be seen that bioactivation moves the curve towards lower concentrations. Figures 50 and 52 show only a modest increase in growth inhibition caused by the metabolic activation of BP.

In summation, metabolic activation of BP decreased the 96-hr EC50(malformation) by 5 to 6 fold. Embryolethality was not affected up to the maximum soluble concentration. Thus, bioactivation significantly increased the potential hazard of BP. Unactivated BP induced primarily gut, mouth, and skeletal malformations (Plate 18). Bioactivation increased the severity of skeletal deformities and caused serious brain (microencephaly) and eye malformations at low BP concentrations. Some of the malformations induced by BP in mammalian test systems were similar to those observed with Xenopus. Shum et al. (46) found that B-naphthoflavone-enhanced BP metabolism in AKR inbred mice injected with BP between 50 and 300 mg/kg was associated with increased in utero toxicity and terata (club foot, cleft palate and lip, kinky tail, hemangioma, anopthalmia and scoliosis). Skeletal kinking in Xenopus may bear some relationship to skeletal limb defects in mammals. Similar terata were also observed in B6 (46) and C57BL/6 (47) strain mice after i.p. injection, but occurred more frequently than in the AKR strain. Greater incidence of anomalies found in the B6 strain has been attributed to genetic variability in the rate of BP biotransformation (i.e. C57BL/6N and B6 strain mice- a highly inducible P-450 isozyme (AAH) responsible for BP metabolism).

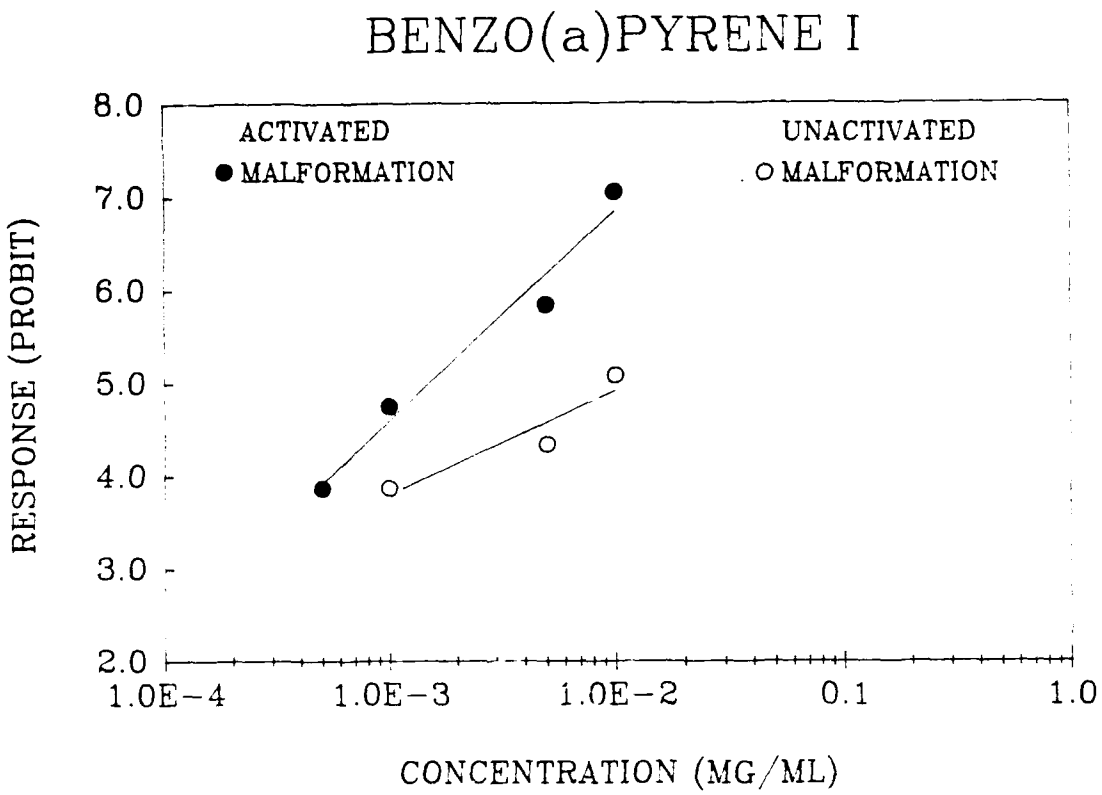

Figure 49 . 96-h Mortality and Malformation Dose-Response Curves for Benzo(a)pyrene Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

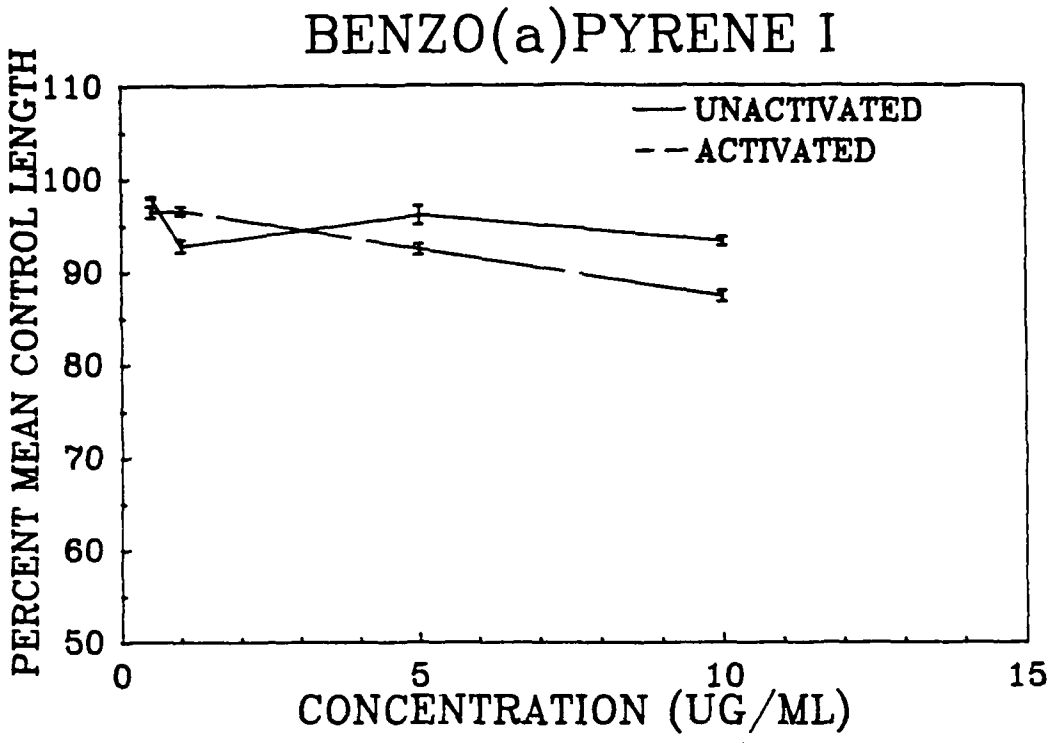

Figure 50. 96-h Growth Dose-Response Curve for Benzo(a)pyrene, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

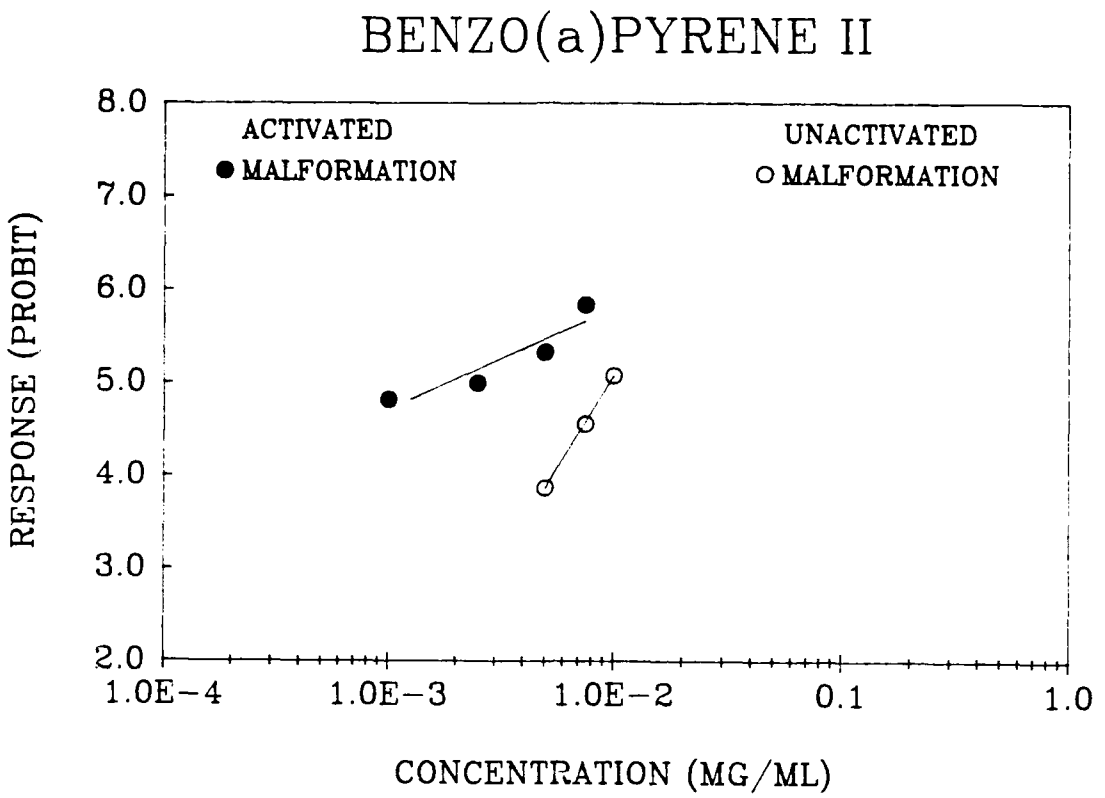

Figure 51. 96-h Mortality and Malformation Dose-Response Curves for Benzo(a)pyrene , Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

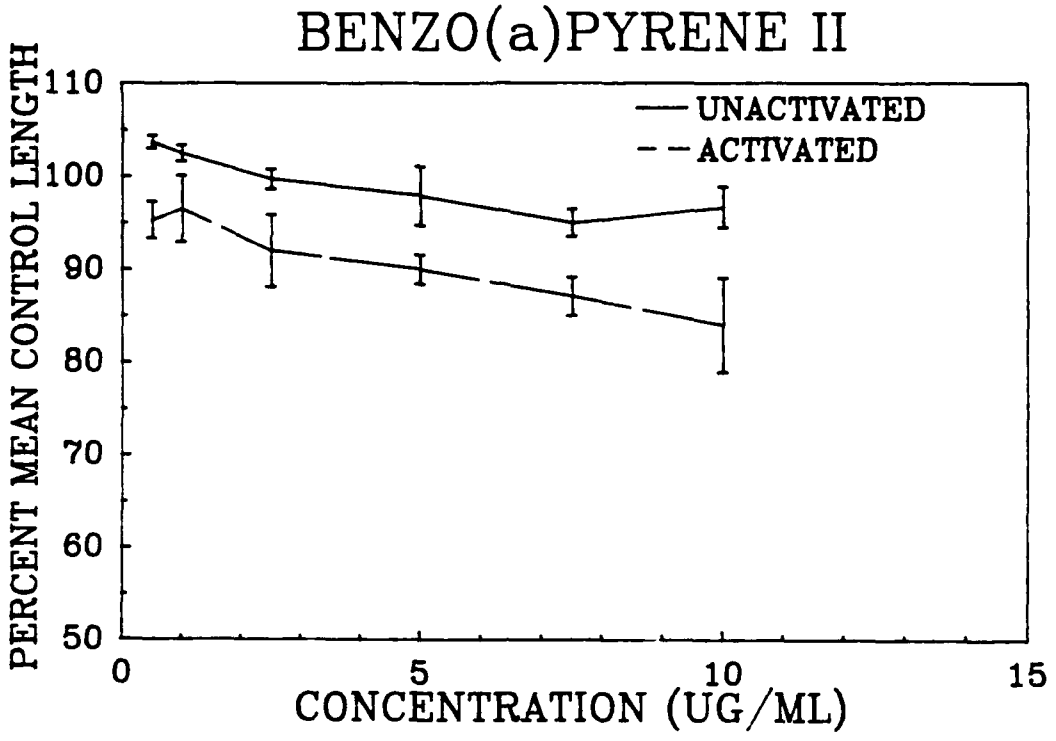

Figure 52. 96-h Growth Dose-Response Curve for Benzo(a)pyrene, Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

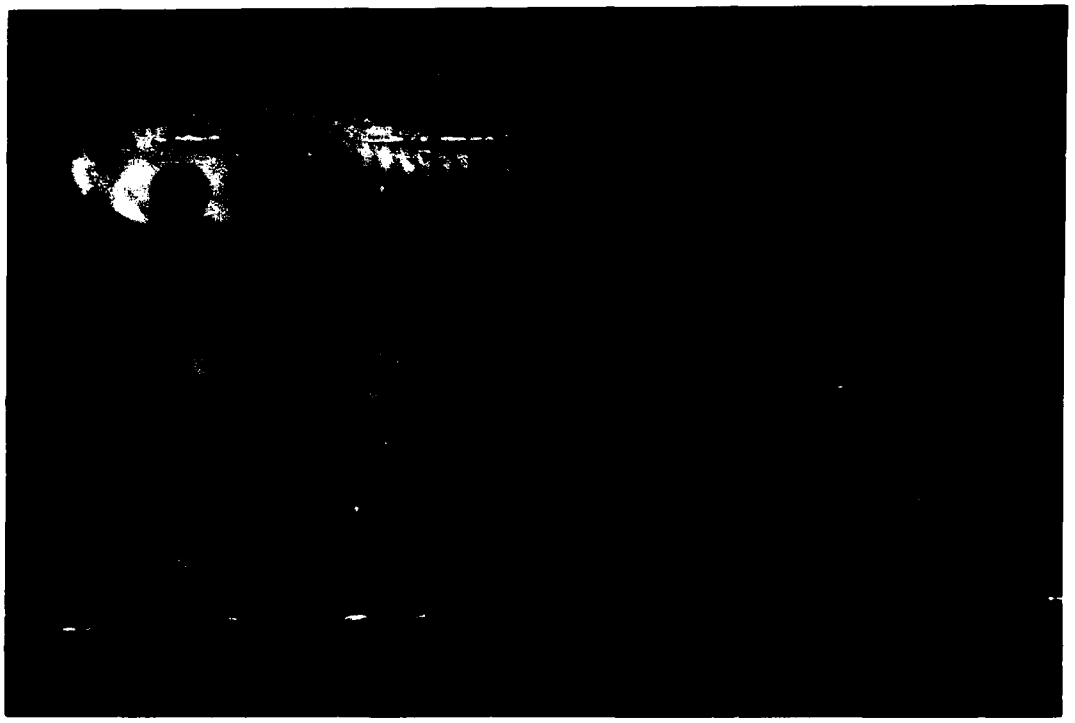

Plate 18. Effects of Different Concentrations of Benzo(a)pyrene on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 10 ug/ml unactivated, 5 ug/ml activated.

Dimethylnitrosoamine: Dimethylnitrosoamine (DMN) is a metabolically activated DNA alkylating agent that possesses considerable clastogenic (48), mutagenic (49) and teratogenic potential (50). DMN is probably metabolized to hydroxymethyl-methylnitrosamine. This developmentally toxic intermediate is very short lived. If the route of injection in mice is such that the dam can detoxify the substance before it gets to the embryo, then little teratogenicity occurs (50). When the route of injection is such that the DMN metabolite can be formed and promptly delivered to the embryo then it is a potent teratogen. This fact poses an unusual problem with FETAX. Since there is no maternal relationship and the metabolite should be formed just outside the Xenopus embryo by the rat liver microsomes, we would predict that unactivated DMN would be relatively nonteratogenic and that metabolized DMN would be about as teratogenic as Benzo(a)pyrene. This would be a perfectly logical finding but nonetheless a false positive result depending on the mammalian route of exposure. This points out the danger of using the mammalian database as a validation standard and the need to consider the metabolism of a toxicant before making a conclusion as to its hazard to human populations.

We have performed only a single test to date on DMN (Table 3). The results indicate that our original prediction is correct in the case of unactivated DMN. The TI is only 1.6 while the 96-hr LC50 and EC50(malformation) is 3.5 and 2.3 mg/ml respectively. Figure 53 shows the 96-hr dose-response curve for mortality and malformation. These curves are reasonably close together. It must be remembered that the 96 hr Xenopus embryo is attaining a degree of metabolic competence during the last stages of development. Thus, the two curves may be slightly wider as a result. Figure 54 shows the growth inhibition curve for DMN. Most of the growth inhibition observed occurs after 50% of the 96-hr LC50 concentration and after this point, the decline is quite sharp to 70% of control values. This growth inhibition curve is typical for a weak teratogen or growth inhibitor. By the next quarterly report we will have micrographs of the types of abnormalities and we will have evidence as to whether MAS causes a significant increase in developmental toxicity. If there is no significant increase then we will try an analog (Acetoxymethyl-Methylnitrosamine) which resembles the active intermediate. If FETAX responds to high doses of this intermediate then we must assume that DMN is quickly degraded on the outside by the rat liver microsomes and that the metabolite does not get into the embryo. If we do not get a response with this analog then we must assume that it either is not getting in or the embryo is not reacting to it.

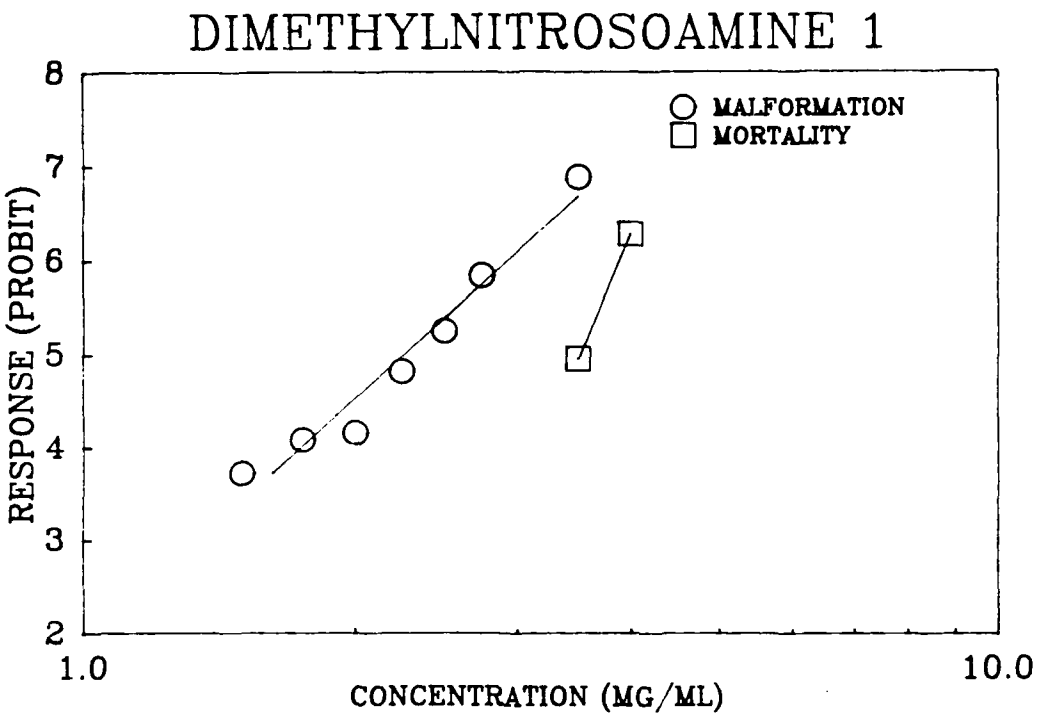

Figure 53. 96-h Mortality and Malformation Dose-Response Curves for Dimethylnitrosoamine, Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

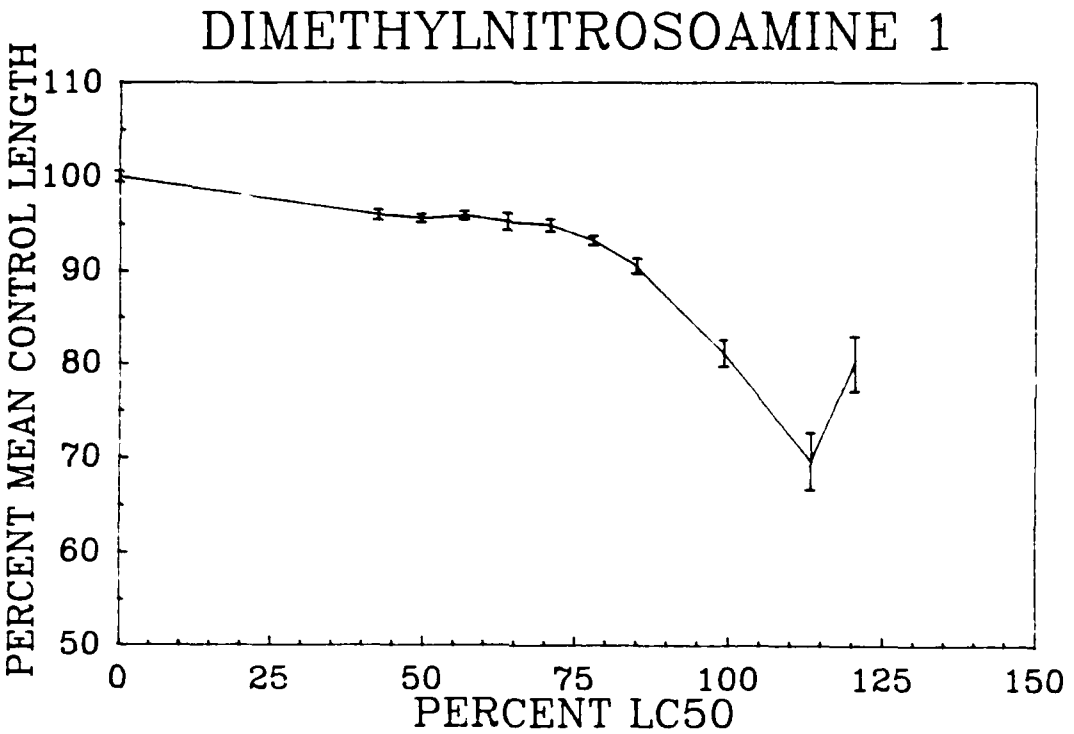

Figure 54. 96-h Growth Dose-Response Curve for Dimethylnitrosoamine, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

Sodium Salicylate: Sodium Salicylate is listed as a variable positive that should not undergo any metabolic activation (Table 3). There is little evidence presented in Shepard (34) that Sodium Salicylate causes terata in humans but it does cause abnormal development in many animal models. Smith et al. (22) list Aspirin, a related compound, as a strong animal positive teratogen but that there is no demonstrated teratogenicity in humans. There is also some variability in the literature on whether biotransformation plays a significant role in teratogenesis. Smith et al. (22) indicated that Aspirin is subjected to hydrolysis and detoxification by conjugation. Gabrielsson and Larsson (51) agree that it is the parent compound (in this case Salicylic Acid) that causes the teratogenic action and not its three major metabolites which do not cause harm when administered in pure form in rats. This data suggests that if biotransformation and conjugation is functional, the embryotoxicity, teratogenicity and growth inhibition of Sodium Salicylate should decrease. However, Juchau (52) has used Sodium Salicylate in the in vitro rat embryo culture system. This is the closest mammalian test system to FETAX plus rat liver microsomes because rat embryos can be cultured with and without S9 supernatant (A source of cytochrome P-450 enzymes). He reports that the addition of S9 to the culture system made little difference in the generation of teratogenicity in vitro. Because of this report we listed Sodium Salicylate as an expected negative for MAS in Table 3.

We found that the mean TI for Sodium Salicylate without MAS was 1.64 and the mean 96-hr LC50 and EC50(malformation) were 2.32 and 1.47 ug/ml respectively (Table 3). With the inclusion of a MAS to the basic FETAX protocol the mean TI dropped slightly to 1.55 and the mean 96-hr LC50 and EC50(malformation) were 2.25 and 1.45 ug/ml respectively. After an examination of the confidence limits for these dose-response curves (see: Data Summary sheets), we concluded as did Juchau (52) that the inclusion of a metabolic activation system had little effect on results. We were disappointed in seeing that the teratogenicity of Sodium Salicylate was very weak as the unactivated TI was only 1.64. However, Sabourin and Faulk (14) listed Aspirin as <1.6 and Dumont obtained a TI value of 1.43 for Sodium Salicylate so we think that our value is correct. Figures 55 and 57 show the dose-response curves for the two tests on this compound. The tests for activated Sodium Salicylate were more repetitive than for unactivated. Figures 56 and 58 show the effect of Sodium Salicylate on embryonic growth. Once again the inclusion of MAS makes little difference in Figure 56 and a greater difference in the higher concentration regions of Figure 58. The inability to cause significant growth inhibition at concentrations below 50% of the 96-hr LC50 is more suggest of a weak or nonteratogen than a strong teratogen. Plate 19 shows that malformations caused by Sodium Salicylate can be quite severe. Whether exposed to Sodium Salicylate with MAS or without, similar types of malformations are observed. Blistering, edema and severe malformations to all major body organs are evident. However, tail kinking is minimal. We have thus far concluded that Sodium Salicylate is not detoxified well in our system and obviously it is not eliminate as it is in mammals. We do a better job of repeating the cultured rat embryo test system than we do the in vivo model.

In summary, our results so far using rat liver microsomes are encouraging. It should be possible to reduce the number of false positives and negatives in FETAX by employing Aroclor 1254 induced rat liver microsomes as an in vitro metabolic activation system. Given that these microsomes are only a subset of all the mammalian detoxification systems, there will be some compounds that still do not test as expected.

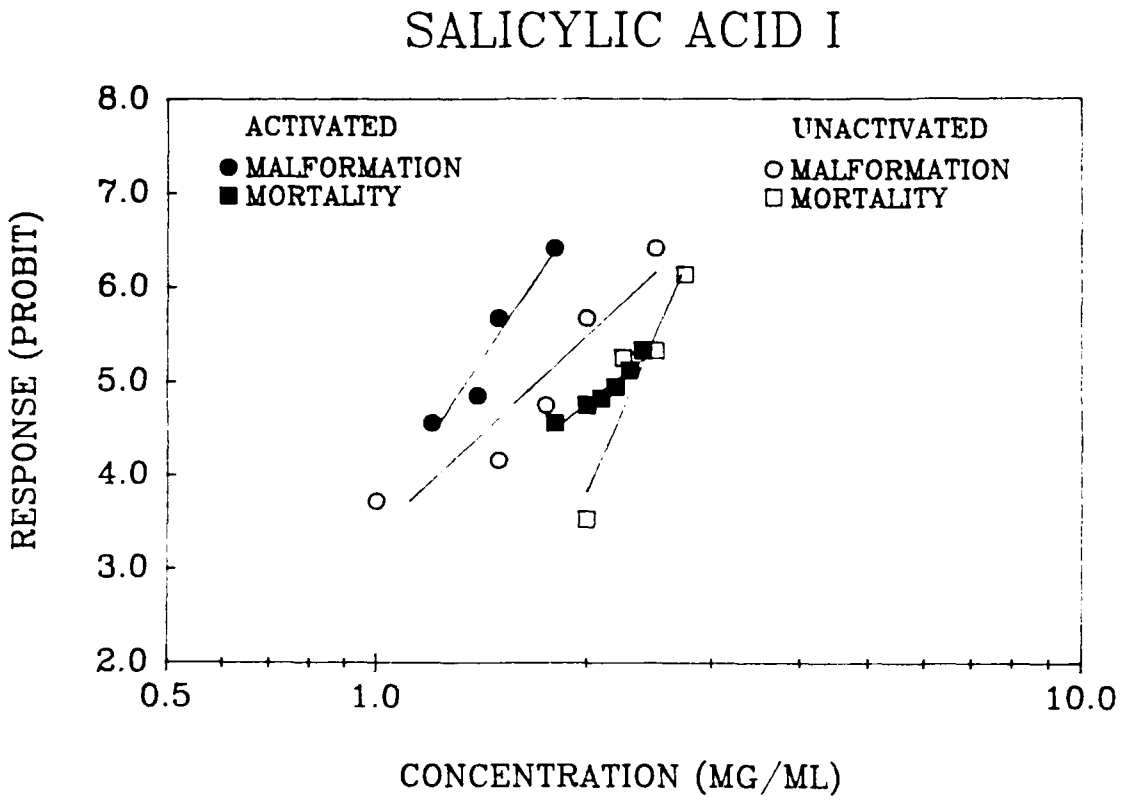

Figure 55. 96-h Mortality and Malformation Dose-Response Curves for Salicyclic Acid, Definitive Test #1. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

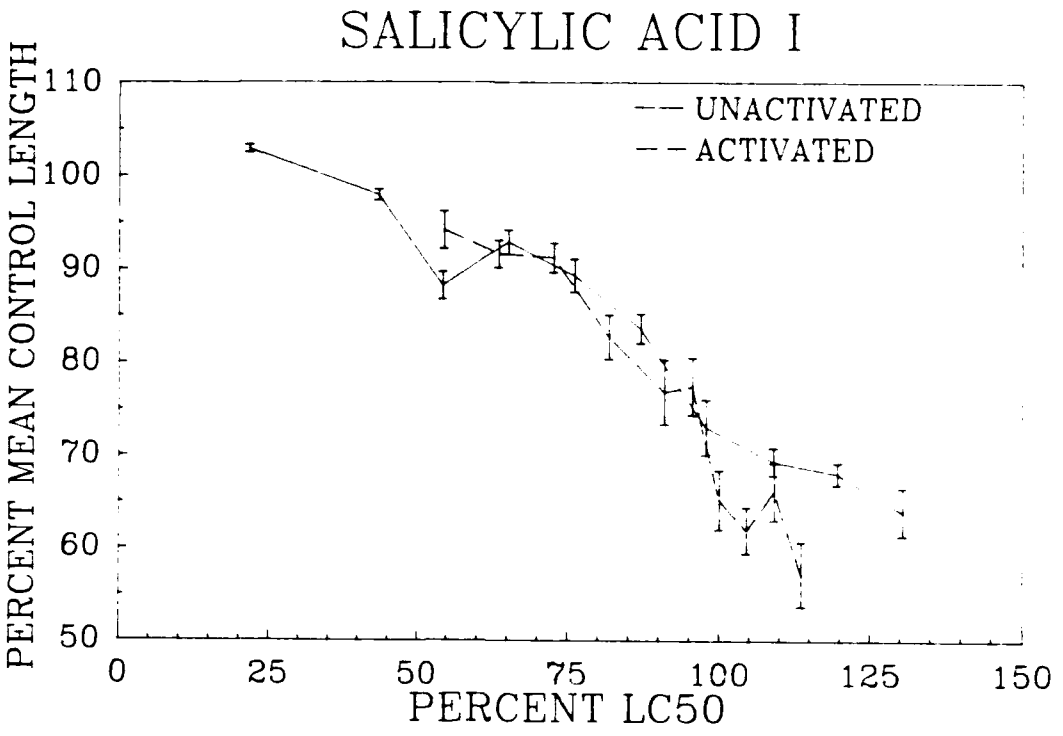

Figure 56. 96-h Growth Dose-Response Curve for Salicyclic Acid, Definitive Test #1. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

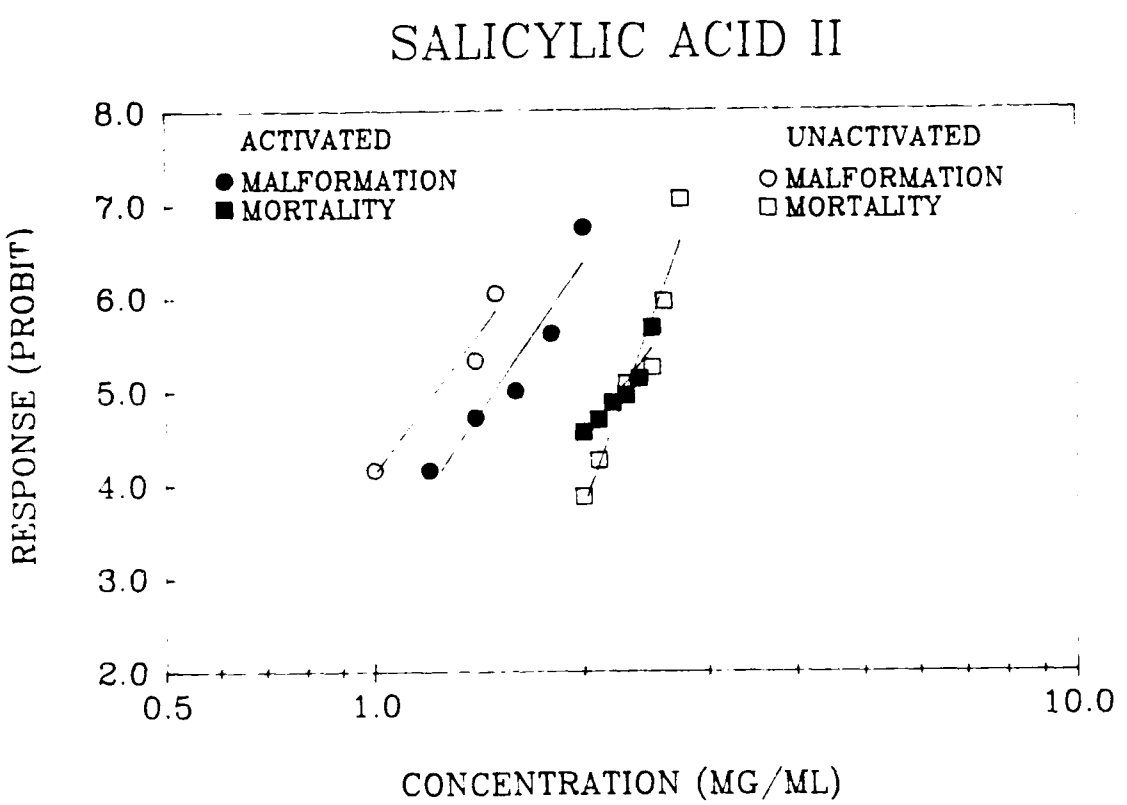

Figure 57. 96-h Mortality and Malformation Dose-Response Curves for Salicyclic Acid, Definitive Test #2. The curve includes only those points used in producing the dose-response curve although other concentrations were tested as well. Each point represents 50 embryos for each concentration.

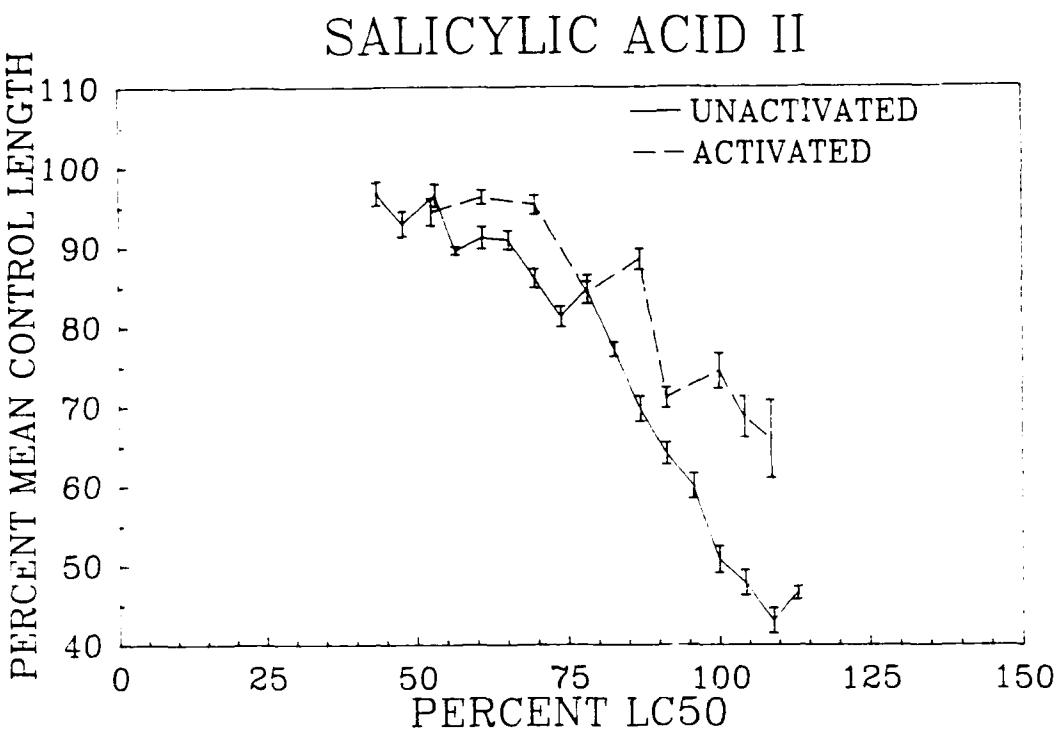

Figure 58. 96-h Growth Dose-Response Curve for Salicyclic Acid, Definitive Test #2. The plot shows the % of control versus the % of the 96-h LC50. Strong teratogens often cause reduction in growth at very low percentages and the slope of the line is very steep. A strong teratogen can cause embryos to become only 45% of control length at high concentrations.

Plate 19. Effects of Different Concentrations of Sodium Salicylate on Xenopus Development. Control embryos achieved Stage 46 at the end of the 96 h exposure period. Side view presented to show effect on brain, eye and spinal cord. From top to bottom: control, 2 ug/ml unactivated, 2 ug/ml activated.

Literature References

1. Dumont, J. N., T. W. Schultz, M. Buchanan, and G. Kao. Frog embryo teratogenesis assay: Xenopus (FETAX)--A short-term assay applicable to complex environmental mixtures. In Waters, Sandhu, Lewtas, Claxton, Chernoff, and Nesnow: "Short-Term Bioassays in the Analysis of Complex Environmental Mixtures III." New York: Plenum Publishing, pp. 393-405, 1983.
2. Goss, L. B., and T. D. Sabourin. Utilization of alternative species for toxicity testing: an overview. J. Appl. Toxicol. 5:193-219, 1985.
3. Best, J. B., and M. Morita. Planarians as a model system for in vitro teratogenesis studies. Terat. Carcin. Mutagen. 2:277-291, 1982.
4. Schuler, R., B. D. Hardin, and R. Niemeier. Drosophila as a tool for the rapid assessment of chemicals for teratogenicity. Terat. Carcin. Mutagen. 2:293-301, 1982.
5. Greenberg, J.. Detection of teratogens by differentiating embryonic nerual crest cells in culture: evaluation as a screening system. Terat. Carcin. Mutagen. 2:319-323, 1982.
6. Greenhouse, G.. Effects of pollutants in embryos and larvae of frogs: a system for evaluating teratogenic effects of compounds in freshwater environments. In "Proceedings of Sixth Annual Conference of Environmental toxicology." National Technical Information Service, pp. 493-511, 1975.
7. Greenhouse, G.. The evaluaiton of toxic effect of chemicals in fresh water by using frog embryos and larvae. Environ. Contam. Toxicol. 20:93-95, 1978.
8. Kimmel, G. L., K. Smith, D. M. Kochar, and R. M. Pratt. Overview of in vitro teratogenicity testing: aspects of validation and application to screening. Terat. Carcin. Mutagen. 2:211-229, 1982.
9. Courchesne, C. L., and J. A. Bantle. Analysis of the activity of DNA, RNA, and protein systhesis inhibitors on Xenopus embryo development. Terat. Carcin. Mutagen. In Press.
10. Sabourin, T. D., R. T. Faulk, and L. B. Goss. The efficacy of three non-mammalian test systems in the identification of chemical teratogens. J. Appl. Toxicol. 5:227-233, 1985a.
11. Dawson, D. A., C. A. McCormick, and J. A. Bantle. Detection of teratogenic substances in acidic mine water samples using the Frog Embryo Teratogenesis Assay--Xenopus (FETAX). J. Appl. Toxicol. 5:234-244, 1985.
12. Dumont, J. N., and T. W. Schultz. Effects of coal-gasification sour water on Xenopus laevis embryos. J. Environ. Sci. Health. A15:127-138, 1980.
13. Sabourin, T. D., R. T. Faulk, and L. B. Goss. Xenopus embryos as teratogen screens: assays with NTP repository chemicals. Soc. Environ. Toxicol. Chemistry, Annual meeting (Abstract). 1985b.
14. Sabourin, T. D., and R. T. Faulk. Comparative evaluation of a short-term test for developmental effects using frog embryos. In: Branbury Report 26: Developmental Toxicology: Mechanisms and Risk pp 203-223, 1987.
15. Johnson, E. M., R. M. Gorman, E. G. Bradley, and M. E. George. The Hydra attenuata system for detetion of teratogenic hazards. Terat. Carcin. Mutagen. 2:263-276, 1982.
16. Clapper, M.L., M.E. Clark, N.W. Klein, P.J. Kurtz, B.D. Carlton and R.S. Chnabra. Cardiovascular defects in rat embryos cultured on serum from

rats chronically exposed to phenytoin. Terat. Carcin. Mutagen. 6:151-161, 1986.
17. Dawson, D.A., and Bantle, J.A., Development of a reconstituted water medium and initial validation of FETAX. Journal of Applied Toxicology. In press, 1987.
18. Wesolowski, M.H. and Lyerla, T.A., The developmental appearance of hexokinase and alcohol dehydrogenase in Xenopus laevis. J. Exp. Zool. 210:211-220, 1979.
19. Fort, D.J., Dawson, D.A., and Bantle, J.A., Development of a metabolic activation system for the frog embryo teratogenesis assay: Xenopus (FETAX). Teratogenesis, Carcinogenesis and Mutagenesis 8(5):251-263, 1988.
20. Kitchin, K. T. and J. S. Woods. 2,3,7,8-Tetrachlorodibenzo-p-dioxin induction of aryl hydrocarbon hydroxylase in female rat liver. Evidence for de novo synthesis of cytochrome P-448. Mol. Pharmacol. 14:890-899, 1978.
21. Bantle, J.A., and Dawson, D.A., Uninduced rat liver microsomes as an in vitro metabolic activation system for the Frog Embryo Teratogenesis Assay -Xenopus (FETAX). Proceedings of the 10th Aquatic Toxicology Symposium, June, (STP-971) pp 316-326, 1988.
22. Smith, M. K., G. L. Kimmel, D. R. Kochar, T. H. Shepard, S. P. Spielberg, and J. G. Wilson. A selection of candidate compounds for in vitro teratogenesis test validation. Terat. Carcin. Mutagen. 3:461-480, 1983.
23. Fantel, A. G., J. C. Greenaway, M. R. Jachau, and T. H. Shepard. Teratogenic bioactivation of cyclophosphamide in vitro. Life Sci. 25:67-72, 1979.
24. Kitchin, K. T., B. P. Schmid, and M. K. Sanyal. Teratogenicity of cyclophosphamide in a coupled microsomal activating/embryo culture system. Biochem. Pharmacol. 30:59-64, 1981.
25. Mirkes, P. E. Cyclophosphamide teratogenesis: A review. Teratogenesis, Carcinogenesis and Mutagenesis. 5:75-88, 1985.
26. Fort, D.J., James, B.L. and Bantle, J.A., Evaluation of the developmental toxicity of five compounds with the frog embryo teratogenesis assay: Xenopus (FETAX). Journal of Applied Toxicology, Accepted June, 1989.
27. Dawson, D.A., Fort, D.J., Smith, G.L., Newell, D.L. and Bantle, J.A., Comparative evaluation of the developmental toxicity of nicotine and cotinine with FETAX. Teratogenesis, Carcinogenesis and Mutagenesis, 8(6):329-338, 1988.
28. Fort, D.J., and Bantle, J.A., Use of frog embryo teratogenesis assay: Xenopus (FETAX) and a exogenous metabolic activation system to evaluate the developmental toxicity of diphenylhydantoin. Fundamental and Applied Toxicology, Submitted June, 1989.
29. NRC. Models for biomedical research: a new perspective. Committee on Models for Biomedical Research, Board on Basic Biology, Commission of Life Sciences, National Research Council. National Academy Press, Washington, DC, 1985, 180 pp.
30. Office of Technology Assessment. U.S. Congress. Alternatives to animal use in testing, research, and education.
31. Shepard, T. H., A. G. Fantel, P. E. Mirkes, J. C. Greenaway, E. Faustman-Watts, M. Campbell, and M. R. Jachau. Teratology testing: I. Development and status of short-term prescreens. II. Biotransformation of teratogens as studied in whole embryo culture. In: Developmental Pharmacology. Eds. S. M. MacLeod, A. B. Okey, and S. P. Spielberg. Raven Press, New York, New York, pp. 147-164, 1983.

32. Johnson, E.M., "A review of advances in prescreening for teratogenic hazards". In: Progress in Drug Research 29:121-154, 1987.
33. Brown, N.A. "Teratogenicity Testing in vitro: Status of validation studies". In: Mechanisms and Models in Toxicology. Arch. Toxicol. Suppl. 11:105-114, 1987.
34. Shepard, T.H., Catalog of Teratogenic Agents. 5th Ed. The Johns Hopkins University Press, Baltimore.
35. Dawson, D.A., and Bantle, J.A., Co-administration of methylxanthines and inhibitor compounds potentiates teratogenicity in Xenopus embryos. Teratology. 35:221-227, 1987.
36. Schmahl, W., Torok, P. and Kreigel H., Embryotoxicity of 5-azacytidine in mice. Phase- and dose-specificity studies. Arch. Toxicol. 55:143-7, 1984.
37. Nelson, B.K., Brightwell, W.S., Setzer, J.V., and O'Donohue, T.L., Reproductive toxicity of the industrial solvent 2-ethoxyethanol in rats and interactive effects of ethanol. Environ. Health Perspect. 57:255-259, 1984.
38. Demey, H.E., Daelemans, R.A., Verpooten, G.A., De-Broe, M.E., and Van-Campenhout, C.M., Propylene glycol-induced side effects during intravenous nitroglycerin therapy. Intensive Care Med. 14:221-226, 1988.
39. Gichner, T., and Veleminsky, J., The organic solvents Acetone, Ethanol, and dimethylformamide potentiate the mutagneic activity of N-methyl-N'-nitro-N-nitrosoguanidine, but have no effect on the mutagenic potential of N-methyl-N-nitrosourea. Mutat. Res. 192:31-35, 1987.
40. Weyland, E.H., and Bevan, D.R., Benzo(a)pyrene disposition in rats following intratracheal instillation, Cancer Res. 46:5655-5661, 1986.
41. Charbonneau, M., Olekevich, S., Brodeur, J. and Plaa, G.L., Acetone potentiation of rat liver injury induced by trichloroethylene-carbon tetrachloride mixtures. Fundam. Appl. Toxicol. 6:654-661, 1986.
42. Caujolle, F.M.E., Caujolle, D.H., Cros, S.B. and Calvet, M.J., Limits of toxic and teratogenic of dimethyl sulfoxide. Ann. N.Y. Acad. Sci. 141:110-125, 1967.
43. Juma, M.B. and Staple, R.E., Effect of maternal administration of dimethylsulfoxide on the development of rat fetuses. Proc. Soc. Exp. Biol. Med. 125:567-569, 1967.
44. Potter, W.Z., Davis, D.C., Mitchell, J.R., Jollow, D.J., Gillette, J.R. and Brodie, B.B., Acetaminophen-induced hepatic necrosis. II. Cytochrome P-450-mediated covalent binding in vitro. The Journal of Pharmacology and Experimental Therapeutics 187:203-210, 1973.
45. Borgen, A., Darvey, H., Castagnoli, N. Crocker, T.T., Rasmussen, R.E. and Yang, I.Y.. Metabolic conversion of Benzo(a)pyrene by syrian hamster liver microsomes and binding of metabolites to deoxyribonucleic acid. Journal of Medicinal Chemistry 16:502-510, 1973.
46. Shum, S., Jensen, N.M., and Nebert, D.W., The murine Ah locus: In utero toxicity and teratogenesis associated with genetic differences in benzo(a)pyrene metabolism. Teratology 20:365-376, 1979.
47. Lambert, G.H. and Nebert, D.W., Genetically mediated induction of drug-metabolizing enzymes associated with congenital effects in the mouse. Teratology 16:147-153, 1977.
48. Sharma, R.K., Jacobson-Kramn, Lemmon, M. and Bakke, J., Galperin, I. and W.F. Blazak, Sister-chromatid exchange and cell replication kinetics in fetal and maternal cells after treatment with chemical teratogens. Mutation Research 158:217-231, 1985.
49. Czygen, P., Greim, H., Garro, A.J., Hutterer, Schaffner, F., Popper, H., Rosenthal, O. and David Y. Cooper. Cancer Research 33:2983-2986, 1973.

50. Bochert, G., Platzek, T. and Neubert, D., DNA modification in murine embryos: a primary cause of embryotoxic effects. In Parmacokinetics in Teratogenesis. Vol II, Ed. H. Nau and W.J. Scott. pp 74-81, 1987.
51. Gabielsson, J. and Larsson., K., The use of physiological pharmokinetic models in studies on the disposition of salicylic acid in pregnancy. In Parmacokinetics in Teratogenesis. Vol II, Ed. H. Nau and W.J. Scott. pp 13-26, 1987.
52. Juchau, M.R. Whole embryo culture: Preinduction in vivo and metabolizing activity in vitro. In Parmacokinetics in Teratogenesis. Vol II, Ed. H. Nau and W.J. Scott. pp 121-132, 1987.

APPENDIX I

Methods of Procedure

SECTION 1.

PREPARATION AND USE OF RAT LIVER MICROSOMES AS A METABOLIC ACTIVATION SYSTEM

MATERIALS AND METHODS

Chemicals and Biochemicals

All chemicals used in rat liver microsome preparation and FETAX were purchased from Sigma (St. Louis, MO.) except for Aroclor 1254 which was obtained from Monsanto (St. Louis, MO.).

Rat Liver Microsome Preparation

Five days prior to microsome preparation, an adult Sprague-Dawley (S/D) strain male rat (200-280 g) was injected with 500 mg/Kg Aroclor 1254 in corn oil (1). A modified procedure for microsomal isolation described by Kitchin and Woods (2) was followed. The liver was perfused with 50 ml of 1.15% KCl containing 0.02 M Tris-HCl buffer, pH 7.5. Homogenization was performed in 1.15% KCl containing 0.02 M Tris-HCl buffer and 0.5% bovine serum albumin (BSA). The homogenate was centrifuged successively at 600 and 9000 x g avg. The crude S-9 supernatant was further purified by two additional ultracentrifugation steps at 102,000 x g avg. The microsomal pellet was then resuspended in 0.05 M Tris-HCl buffer, pH 7.5, put into 1 ml aliquots, and immediately frozen in liquid nitrogen (3). Protein was determined by the method of Bradford (4) (BioRad, Richmond, CA). P-450 activity was inferred by the measurement of formaldehyde generated from the N-demethylation of aminopyrine by the method of Nash (5). Assay conditions were described by Lucier et al. (6). Several aliquots of microsomes were chemically reduced with dithionite and pretreated with carbon monoxide (CO) to selectively inactivate P-450 activity (7).

Animal Care and Breeding

Xenopus adult care, breeding, and embryo collection was performed according to Courchesne and Bantle (8). Initial tests were conducted to optimize the cytochrome P-450 activity and determine the most suitable concentration of microsomal protein, NADPH generating system, and antibiotics using sublethal and subteratogenic levels of unactivated CP. For each separate clutch of embryos, four sets of 20 were placed in 60 mm covered plastic Petri dishes containing FETAX solution, a reconstituted water media (9), and 100 U/ml penicillin-100 ug/ml streptomycin in 8 ml of total solution. These were designated FETAX controls. Treatments were performed in duplicate with 20 embryos per dish. Each test dish received microsomal protein, NADPH generating system, 100 U/ml penicillin-100 ug/ml streptomycin, and various concentrations of CP. The NADPH generating system consisted of 3.5 mM glucose 6-phosphate, 0.31 U/ml glucose 6-phosphate dehydrogenase, 0.1 mM NADP, and 0.007 mM NADPH. Metabolic activation system, unactivated CP controls, and CO-gassed microsomes and cyclophosphamide were prepared in a similar manner. All dishes contained 8 ml of the appropriate solutions, which were previously diluted from stocks prepared in FETAX solution. All solutions were removed every 24 h of the four d test and fresh solutions added in a static-renewal fashion.

Data Collection and Analysis

Dead embryos were removed every 24 h and the number recorded. Death at 24 and 48 h was ascertained by embryo skin pigmentation, structural integrity,

and irritability. At 72 and 96 h the lack of a heart beat was an unambiguous sign of death in the transparent embryo. Surviving larvae were fixed in 0.6% formalin, pH 7.0. The number of live-malformed embryos and the stage of development according to Nieuwkoop and Faber (10) were determined. Dose-response bioassays evaluated according to Litchfield-Wilcoxon (11) were used to determine the 96 h LC50 and EC50 (malformation). The 96 h LC50 was divided by the EC50 (malformation) yielding a teratogenicity index (TI) which has proven useful in assessing teratogenic risk (12, 13). Head-tail length was measured using a IBM AT equivalent computer, a Kurta digitizing pad and Sigma Scan software.

References

1. Freireich, E.J., Quantitative comparison of toxicity of anti-cancer agents in mouse, rat, dog, monkey, and man. Cancer Chemother Rep 50:219-244, 1977.

2. Kitchin K.T., and J.S. Woods, 2,3,7,8- Tetrachlorobenzo-p-dioxin induction of aryl hydrocarbon hydroxylase in female rat liver, evidence for de novo synthesis of cytochrome P-450. Mol. Pharmacol. 14:890-899, 1978.

3. Dent, J.G., S. Schnell, Graichen, M.E., P. Allen, Abernathy, D., D.B. Couch. Stability of activating systems for in vitro mutagenesis assays: Enzyme activity and activating ability following long-term storage at -85°C. Environ. Mutagen. 3:167-179, 1981.

4. Bradford, M.M., A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254, 1976.

5. Nash, T.. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochemistry 55:412-416, 1955.

6. Lucier, G., O. McDaniel, P. Brubaker and R. Klein, Effects of methymercury hydroxide of rat liver microsomal enzymes. Chem. Biol. Interact. 4:265-280, 1971.

7. Bantle, J.A., and Dawson, D.A., Uninduced rat liver microsomes as an in vitro metabolic activation system for the Frog Embryo Teratogenesis Assay -Xenopus (FETAX). Proceedings of the 10th Aquatic Toxicology Symposium, June, (STP-971) pp 316-326, 1988.

8. Courchesne, C.L., and Bantle, J.A., Analysis of the activity of DNA, RNA and protein synthesis inhibitors on Xencpus embryo development. Teratogenesis, Carcinogenesis and Mutagenesis 5:177-193, 1985.

9. Dawson, D.A., and Bantle, J.A., Development of a reconstituted water medium and initial validation of FETAX. Journal of Applied Toxicology. 7:237-244, 1987.

10. Nieuwkoop, P.D. and J. Faber, "Normal table of Xenopus laevis (Daudin). " 2nd ed. Amsterdam: North Holland, 1975.

11. Litchfield, J.T. and F. Wilcoxon, A simplified method for evaluating dose-response expewriments. J. Pharmacol. Exp. Ther. 96:99-113, 1949.

12. Dumont, J., T M. Schultz, M. Buchanan and G. Kao, Frog Embryo Teratogenesis Assay- Xenopus (FETAX)- A short-term assay applicable to complex environmental mixtures. In Waters, M.D., S.S. Sandhu, J. Lewtas, L. Claxton and S. Nesnow.: "Short-term Bioassays in th Analysis of Complex Environmental Mixtures, III. " New York: Plenum Publishing, 1983, pp 393-405.

13. Dawson, D.A., and Bantle, J.A., Co-administration of methylxanthines and inhibitor compounds potentiates teratogenicity in Xenopus embryos. Teratology. 35:221-227, 1987